Polo-Like Kinase 4 (PLK4) Function in Cancer Progression

by

Karineh Kazazian

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Institute of Medical Science
University of Toronto

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Abstract
Invasion and metastasis are responsible for the majority of deaths from cancer, but the molecular mechanisms that facilitate execution of the metastatic cascade remain incompletely understood. One strategy is to identify genes that drive the metastatic process, and then target these drivers with biologic therapies in either the adjuvant or the metastatic setting. The polo family member polo-like kinase 4 (Plk4), is a serine-threonine kinase that is most highly expressed at the centrosome, where it is a master regulator of centriole duplication. Emerging evidence suggests that increased Plk4 levels in established malignancy may promote tumour progression; high levels of Plk4 expression have been reported in many tumour types, including breast, colorectal and pancreas, and its overexpression has been linked with resistance to therapy and death from cancer. While the critical role played by Plk4 in centriolar biogenesis is well-acknowledged, we recently discovered a novel and unexpected localization of Plk4 to the edges of lamellipodia and filopodia of motile cells, and identified a gene expression pattern predictive of reduced motility in Plk4<sup>+/−</sup> murine embryonic fibroblasts. I functionally validated this prediction in cancer cells, showing enhancement by Plk4 of cancer cell migration and invasion in vitro, and of MDA-MB-231 human breast cancer xenograft progression. Furthermore, this Plk4-driven invasive phenotype correlates with acquisition of an epithelial-to-mesenchymal transition (EMT) profile, cell polarity, spreading and enhanced directional protrusion formation. Investigation of
pathways upstream of actin polymerization revealed that Plk4 regulates activation of the motility-related RhoGTPase Cdc42, and others have demonstrated that Plk4 promotes Rac1 activation, however this was insufficient for Plk4-induced motility in our system. In a BioID screen for Plk4 interactors, members of the Arp2/3 complex were identified, and I confirmed a novel physical and functional interaction between Plk4 and Arp2 in mediating Plk4-driven cancer cell movement. The interaction is mediated through the Plk4 PB1-PB2 domain, and results in phosphorylation of Arp2 at the T237/T238 activation site. These results provide a further rationale for application of targeted anti-Plk4 therapy in the clinical treatment of cancer patients, and reveal a new role for Plk4 in regulating Arp2/3-mediated actin cytoskeletal rearrangement.
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Contributions

The author performed all experiments described in this thesis, except as noted below:

Christopher Go – BioID purification and mass spectrometric analysis (with guidance by Dr. Anne-Claude Gingras)

Dr. Hannah Wu – histopathology of mouse tissues

Roland Xu – Real Time Cell Analyzer (RTCA) invasion assays and spheroid invasion assay

Olga Brashavitskaya – Cdc42-GTP pull-down assay for Figure 5.3b left panel, and artwork for Figure 1.1

Karina Pacholczyk – real time RT-PCR
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<th>Abbreviation</th>
<th>Explanation</th>
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<tbody>
<tr>
<td>ABC</td>
<td>Ammonium bicarbonate</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
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<tr>
<td>Arp</td>
<td>actin related protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Cell division cycle 42</td>
</tr>
<tr>
<td>CDK1</td>
<td>Cyclin-Dependent Kinase 1</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CIN</td>
<td>Chromosomal Instability</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>DAPI</td>
<td>6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-Dimethylbenz[a]anthracene</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra Cellular Matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-Mesenchymal Transition</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin-fixed paraffin-embedded</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>G1</td>
<td>Gap1</td>
</tr>
<tr>
<td>G2</td>
<td>Gap2</td>
</tr>
<tr>
<td>GAP</td>
<td>GTP-ase Activating Protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDI</td>
<td>Guanine Dissociation Inhibitors</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine Exchange Factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>Hand1</td>
<td>Heart And Neural Crest Derivatives Expressed 1</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B Virus</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular Carcinoma</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>LIMK</td>
<td>LIM-kinase</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of Heterozygosity</td>
</tr>
<tr>
<td>M</td>
<td>Mitotic phase</td>
</tr>
<tr>
<td>MDFIC</td>
<td>MyoD Family Inhibitor Domain Containing</td>
</tr>
<tr>
<td>mDia</td>
<td>mouse Diaphanous-related formin</td>
</tr>
<tr>
<td>MEFs</td>
<td>Murine Embryonic Fibroblasts</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen Activating Protein Kinase</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal-to-Epithelial Transition</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinases</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MT</td>
<td>Microtubules</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule Organizing Center</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Neural Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>P</td>
<td>Passage number</td>
</tr>
<tr>
<td>P53</td>
<td>Tumour Protein p53</td>
</tr>
<tr>
<td>PB</td>
<td>Polo-Box Domain</td>
</tr>
<tr>
<td>PBGD</td>
<td>Porphobilinogen deaminase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCM</td>
<td>Pericentriolar Material</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>PI(3,4,5)</td>
<td>Phosphatidylinositol 3,4,5 triphosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositol 3-Kinase</td>
</tr>
<tr>
<td>Plk</td>
<td>Polo-Like Kinase</td>
</tr>
<tr>
<td>Ras</td>
<td>Rat sarcoma viral oncogene homolog 2</td>
</tr>
<tr>
<td>Rac1</td>
<td>Ras-Related C3 Botulinum Toxin Substrate 1</td>
</tr>
<tr>
<td>Rho</td>
<td>Ras homolog gene family member</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated, coiled-coil-containing protein kinase 1</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>S</td>
<td>Synthesis phase</td>
</tr>
<tr>
<td>Sak</td>
<td>Snk Akin Kinase</td>
</tr>
<tr>
<td>SAPK/KK</td>
<td>Stress-activated protein kinase kinase kinases</td>
</tr>
<tr>
<td>Scar/WAVE</td>
<td>Suppressor of cAMP receptor/Wiskott-Aldrich syndrome protein-family verprolin homology protein</td>
</tr>
<tr>
<td>SCF</td>
<td>Skp-Cullin-1-F-Box Proteasome</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short Interfering RNA</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor beta</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-Tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethyl rhodamine iso-thiocyanate</td>
</tr>
<tr>
<td>WASH</td>
<td>Wiskott–Aldrich scar homology</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott–Aldrich Syndrome protein</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
</tr>
<tr>
<td>ZEB1/2</td>
<td>Zinc finger E-box-binding homeobox 1/2</td>
</tr>
</tbody>
</table>
Chapter 1: Literature Review

1.1 The Polo-like kinase (Plk) family: expression, localization and cellular functions

The Polo family consists of five structurally conserved serine-threonine kinases characterized structurally by two or three polo-box motifs at the carboxy-terminus, which play critical roles in cell cycle progression/regulation, response to DNA damage and oxidative stress. Expression and/or activation of the Plks is tightly regulated throughout the cell cycle (Figure 1.1a), with disruption affecting a variety of fundamental cellular processes and leading to genomic instability and potentially carcinogenesis. As cells progress through the various stages of the cell cycle, the Plks localize to different subcellular structures or compartments, allowing them to perform distinct activities (Figure 1.1b; reviewed in (Zitouni, Nabais et al. 2014)).

1.1.1 Plk1

Plk1, which is ubiquitously expressed and the most studied member of the Plk family, is important in coordinating the centrosome cycle with the cell cycle. The centrosome cycle begins with the initiation of centriole duplication in G1/S, which is followed by centrosome maturation in G2 and through to mitosis. At mitotic entry, centrosomes move to the opposite ends of the cell, where they act as part of the microtubule organizing center (MTOC). In turn, the MTOC forms the mitotic spindle complex, which ensures proper segregation of sister chromatids and equal partitioning of DNA to daughter cells. Plk1 expression levels begin to increase in S phase, when it aids the formation of pre-replicative complexes in preparation for DNA replication (Golsteyn, Schultz et al. 1994, Wu and Liu 2008, Song, Liu et al. 2011), and peaks in G2 and mitosis, when it functions in Cdk1/CyclinB activation, leading to promotion of G2/M transition.
At G2 and during the first half of M, Plk1 localizes to the centrosomes and promotes their maturation and separation to the opposite ends of the cell (Lane and Nigg 1996, Joukov, Walter et al. 2014, Kong, Farmer et al. 2014). Plk1 is also a major player in mitotic spindle assembly, microtubule-kinetochore attachment and chromosome segregation (Golsteyn, Schultz et al. 1994, Casenghi, Meraldi et al. 2003, Sumara, Gimenez-Abian et al. 2004, Ahonen, Kallio et al. 2005, Feng, Yuan et al. 2006, Kang, Park et al. 2006, Nishino, Kurasawa et al. 2006, Park, Park et al. 2015). At the final stages of mitosis, Plk1 localizes to the spindle midzone where it serves to ensure accurate positioning of the cleavage furrow and proper cytokinesis (Qian, Erikson et al. 1999, Burkard, Randall et al. 2007).

1.1.2 Plk2, Plk3 and Plk5

Plks 2, 3 and 5 have been described as immediate early response genes as the expression of these three kinases increases upon DNA damage and oxidative stress (Simmons, Neel et al. 1992, Donohue, Alberts et al. 1995, Andrysik, Bernstein et al. 2010, Xu, Yao et al. 2010, Hiremath, Mundaganur et al. 2014, Li, Ma et al. 2014). This in turn leads to the activation of the G2/M checkpoint in a p53 dependent manner (Xie, Wang et al. 2001, Xie, Wu et al. 2001, Burns, Fei et al. 2003, Bahassi el, Myer et al. 2006, Valenti, Fausti et al. 2011). Overexpression or ectopic expression of Plk3 or Plk5 leads to G1 arrest and apoptosis, suggesting a role for these kinases in G1/S transition (Conn, Hennigan et al. 2000, Andrysik, Bernstein et al. 2010). Indeed, Plk3 activity peaks in G1 where it positively regulates G1/S transition and promotes DNA replication (Zimmerman and Erikson 2007). The role played by Plk5 in the DNA damage response and DNA replication remains to be determined. In addition to invoking the DNA damage checkpoint, Plk2 functions in centriole duplication (Warnke, Kemmler et al. 2004). Plk2 phosphorylation of its downstream targets leads to their binding to centriolar proteins and
Figure 1.1: Plks in the cell cycle. Figure from Kazazian, Brashavitskaya et al., 2015. The activity of individual Plks is controlled temporally (a,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 a peak in G2/M. Plk5 is expressed only 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 facilitates DNA synthesis. Plk4 localizes to the centrosomes in interphase and mitotic onset and is essential for centriole duplication. 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 telophase, Plk1, Plk3 and Plk4 localize to midbody where they play an active role in cytokinesis.
contributes to the initiation of centriole biogenesis (Chang, Cizmecioglu et al. 2010, Krause and Hoffmann 2010, Cizmecioglu, Krause et al. 2012). Plk2 is expressed in a tissue-specific manner, with high levels of Plk2 mRNA expression detected in murine brain, heart and lung, and in human brain, heart, uterus, testis, mammary glands, spleen and trachea (Simmons, Neel et al. 1992, Liby, Wu et al. 2001). Plk3 has also been reported to localize to the centrosomes, mitotic spindle and midbody, although its function at the centrosome and during cytokinesis is still unclear (Conn, Hennigan et al. 2000, Wang, Xie et al. 2002, Jiang, Wang et al. 2006). One possibility is that Plk3 may play a role in regulation of microtubular dynamics (Wang, Xie et al. 2002, Wang, Beauchemin et al. 2011). Plk3 is relatively lowly expressed in most adult tissues, with higher levels detected in murine skin, lung and brain and human placenta, ovary and lung (Donohue, Alberts et al. 1995, Li, Ouyang et al. 1996). Plk5 expression is confined predominantly to the brain. Human Plk5 lacks a kinase domain and is involved in neuronal differentiation and formation of neuritic processes (Andrysik, Bernstein et al. 2010, de Carcer, Escobar et al. 2011).

1.1.3 Plk4 in Cell Cycle Progression

Plk4 differs from the other members of the Plk family in that it has a unique central region, which contains two tandem polo boxes (PB1-PB2), in addition to its carboxy-terminal polo box (PB3) (Figure 1.2; (Slevin, Nye et al. 2012)). This triple polo box architecture promotes trans-autophosphorylation of the phosphodegron motif, termed the downstream regulatory element, and degradation of Plk4, thereby restricting centriole duplication to once per cell cycle and helping prevent multipolar spindles and chromosomal instability (Guderian, Westendorf et al. 2010, Holland, Lan et al. 2010, Sillibourne, Tack et al. 2010). Plk4 expression is controlled at both the transcriptional and post-translational levels; Plk4 is regulated and rapidly degraded by
Figure 1.2: Model representation of Plk4 domains, and activation/degradation. 

a) Plk4 is the most structurally divergent member of the Plks, with a triple polo-box (PB) architecture. Similar to other Plk family members it contains an N-terminal kinase domain.

b) Inactive Plk4 homodimerizes through the PB1-PB2 domains, and C-terminal intertwining of the PB3 domains. Plk4 becomes active through trans-autophosphorylation or phosphorylation by another kinase such as the stress-activated protein kinase (SAPK). Trans-autophosphorylation of the Slimb-binding domain (phosphodegron) results in its ubiquitination by the SCF complex (SKP1-cullin-F-box), and subsequent degradation.
the SKP1-Cullin1-F-box (SCF) Slimb/β-TrCP ubiquitin ligase throughout the cell cycle, with both Plk4 protein and mRNA expression increasing gradually in G1 to reach a peak in mitosis (Figure 1.3a; (Fode, Binkert et al. 1996, Cunha-Ferreira, Rodrigues-Martins et al. 2009, Cunha-Ferreira, Bento et al. 2013, Klebba, Buster et al. 2013)). Plk4 expression levels start to rise in S phase, where it functions in centriole biogenesis (Fode, Binkert et al. 1996, Kleylein-Sohn, Westendorf et al. 2007, Eckerdt, Yamamoto et al. 2011), and peaks in mitosis, where it facilitates late mitotic progression (Hudson, Kozarova et al. 2001). A recent study by Zitouni and colleagues (Zitouni, Francia et al. 2016), identified a mechanism coupling cell cycle progression to centriole biogenesis, where in mitosis the mitotic kinase CDK1-CyclinB binds STIL and prevents formation of the Plk4-STIL complex and STIL phosphorylation by Plk4, thereby inhibiting untimely centriole biogenesis. Upon mitotic exit and CDK1-CyclinB inactivation, Plk4 is able to phosphorylate STIL in G1, which recruits HsSas-6 and allows for procentriole assembly in S phase. During cytokinesis, Plk4 localizes to the midbody where it may assist in proper cleavage furrow positioning, though this remains controversial (Hudson, Kozarova et al. 2001, Rosario, Ko et al. 2010, Holland, Fachinetti et al. 2012). In M phase, Plk4 is dephosphorylated by the Protein Phosphatase 2A, which stabilizes Plk4 and allows centriole duplication in D. melanogaster, although a stabilizing phosphatase has not been identified in mammalian cells (Brownlee, Klebba et al. 2011). Plk4 localization to the centrioles is regulated by the binding of the tandem PB1-PB2 to CEP152/Asterless (Cizmecioglu, Arnold et al. 2010, Dzhindzhev, Yu et al. 2010, Hatch, Kulkian et al. 2010) and CEP192 (Kim, Park et al. 2013, Sonnen, Gabryjonczyk et al. 2013, Park, Park et al. 2014); PB3 also confers centriole localization, although it does not bind CEP152/Asterless in vitro (Hudson, Kozarova et al. 2001, Habedanck, Stierhof et al. 2005, Dzhindzhev, Yu et al. 2010). Plk4 distribution along the
Figure 1.3: Plk4 in centriole duplication. a) Plk4 protein and mRNA expression increases gradually in G1 to reach a peak in mitosis. b) CEP192 and CEP 152 weakly interact, and acting as scaffolding proteins CEP192 and CEP152 bind to Plk4 in a hierarchical and competitive fashion to recruit Plk4 to the site of daughter centriole assembly. Plk4 has been shown to bind and be subject to FBXW1/β-TrCP-dependent proteasomal degradation subsequent to autophosphorylation within the phosphodegron motif. In Drosophila S2 cells, PP2A (Protein Phosphatase 2A(Twins)) counteracts SAK autophosphorylation, thus stabilizing it and promoting centriole duplication, although a stabilizing phosphatase has not been identified in mammalian cells. Phosphorylation of STIL by Plk4 facilitates the Sas-6-STIL interaction and centriolar loading of Sas-6, leading to assembly of the centriolar cartwheel structure. In turn, STIL regulates the centriolar distribution of Plk4. Plk4 has also been shown to phosphorylate FBXW5, reducing the activity of the SCF-FBXW5 E3-ubiquitin ligase and suppressing its ability to ubiquitinylate Sas-6. Plk4 expression is regulated in a p53 dependent manner in response to centrosome loss or overduplication. Other interactors reported to be involved in centriolar duplication include CDK11p58, GCP6 a core component of the γ-tubulin ring complex which physically interacts with and is phosphorylated by Plk4, and the endogenous regulator of Plk4 activity FAM46C (unpublished).
centriole is further restricted by STIL localization, and concomitantly HsSas-6, and Plk4’s interaction with STIL may protect centriolar Plk4 protein from degradation (Ohta, Ashikawa et al. 2014). Depletion or inhibition of Plk4 results in cell cycle arrest associated with loss of centrioles in a p53-dependent manner, where repression of p53 allows cells that fail centriole duplication or have increased centrosomes to proliferate indefinitely and overcome the apoptosis resulting from elevated Plk4 expression in the murine brain (Marthiens, Rujano et al. 2013, Bazzi and Anderson 2014, Lambrus, Uetake et al. 2015, Wong, Anzola et al. 2015).

1.1.4 Plk4 in Centriole Duplication

Since 1900, as initially described by Boveri and Hansemann (reviewed in (Scheer 2014)), the centrosome has been identified as the main microtubule organizing centre in mammalian cells, responsible for assembling bipolar mitotic spindles during mitosis. Centrosomes are comprised of a pair of centrioles, nine-fold symmetrical microtubule triplets, surrounded by dense pericentriolar material. In mitosis, two pairs of orthogonally arranged centrioles separate to form the poles of the mitotic spindle, allowing for chromosome segregation. Canonical centriole duplication begins at the G1/S transition, where the key regulator of centriole duplication, Plk4, phosphorylates the centriolar protein, STIL allowing it to interact with Sas-6 and assemble the centriolar cartwheel structure on the wall of the preexisting mother centriole and template the daughter procentriole by providing a scaffold on which microtubules are loaded (Kitagawa, Vakonakis et al. 2011, van Breugel, Hirono et al. 2011, Dzhindzhev, Tzolovsky et al. 2014, Ohta, Ashikawa et al. 2014, Kratz, Barenz et al. 2015, Moyer, Clutario et al. 2015).

Centrosome number is normally tightly controlled within the cell division cycle. Plk4 kinase activity is required for centriole duplication. Plk4 overexpression or elimination of the SCF
ubiquitin-protein ligase required for Plk4 destruction results in centrosomal amplification in which numerous daughter centrioles arise around the pre-existing mother centriole, forming a rosette (Bettencourt-Dias, Rodrigues-Martins et al. 2005, Habedanck, Stierhof et al. 2005, Cunha-Ferreira, Rodrigues-Martins et al. 2009, Rogers, Rusan et al. 2009, Sillibourne, Tack et al. 2010); In some systems, Plk4 overexpression or restoring endogenous levels of Plk4 has also been shown to cause de novo centriole biogenesis in the absence of a template (Peel, Stevens et al. 2007, Rodrigues-Martins, Riparbelli et al. 2007, Lambrus, Uetake et al. 2015, Wong, Anzola et al. 2015). Overduplication of centrosomes and the resultant predisposition to multipolar spindle formation can lead to mitotic errors and chromosomal instability, particularly when cell cycle checkpoints are loosened such as when p53 function is reduced (Fujiwara, Bandi et al. 2005, Lingle, Lukasiewicz et al. 2005, Pelletier 2008, Bazzi and Anderson 2014, Izquierdo, Wang et al. 2014). Centrosome amplification is, in fact, a common feature of cancer cells, with supernumerary centrosomes present in both solid and haematological malignancies, including lymphoma, myeloid malignancies, and pancreatic, colorectal, ovarian and prostate cancers ((Lingle, Lutz et al. 1998, Pihan, Purohit et al. 1998, Sato, Mizumoto et al. 1999, Hsu, Kapali et al. 2005), reviewed in (Kramer, Neben et al. 2005). When noted in advanced stages of disease, centrosome amplification has been identified as a marker of poor prognosis in aggressive, drug-resistant tumours (Glinsky 2006, Finetti, Cervera et al. 2008). Depletion of Plk4 results in reduced centriole number and abnormal mitotic spindles, constituting an alternate mechanism of Plk4-related tumourigenesis (Bettencourt-Dias, Rodrigues-Martins et al. 2005, Ko, Rosario et al. 2005, Li, Tan et al. 2005, Castellanos, Dominguez et al. 2008).

While our understanding of Plk4’s key role early in centriole duplication has improved significantly in recent years, the pathways that control initiation of centriole assembly are, as of
yet, not fully understood. Genetic and genome-wide RNAi screens have identified several proteins in *C. elegans* necessary for canonical centriole assembly, including ZYG-1, considered a functional homolog of Plk4 and a key regulator of centriole duplication. SPD-2, which is the homolog of human CEP192, is necessary to recruit ZYG-1 to the centrosome, and both proteins in turn recruit Sas-4, Sas-5 and Sas-6 (Delattre, Leidel et al. 2004, Pelletier, Ozlu et al. 2004, Kleylein-Sohn, Westendorf et al. 2007). In mammalian cells, several proteins essential to centriole duplication, including Plk4, HsSas-6, Asterless/CEP152, Sas-4/CPAP, Sas-5/STIL, CEP135/BLD10, CP110, and more recently CEP192/SPD-2, have been identified (Figure 1.3b; (Leidel, Delattre et al. 2005, Kleylein-Sohn, Westendorf et al. 2007, Strnad, Leidel et al. 2007, Azimzadeh and Marshall 2010, Kim, Park et al. 2013, Sonnen, Gabryjonczyk et al. 2013)). CEP152/Asterless recruits Plk4 to the site of daughter centriole assembly, acting as a scaffolding protein, however depletion of CEP152 does not significantly decrease Plk4 levels at the centrosome (Cizmecioglu, Arnold et al. 2010, Dzhindzhev, Yu et al. 2010, Hatch, Kulukian et al. 2010). More recently it has been shown that CEP192 and CEP152 cooperate in the centrosomal recruitment of Plk4, and also cooperate in centriole duplication (Kim, Park et al. 2013, Sonnen, Gabryjonczyk et al. 2013). CEP192 and CEP152 weakly interact, and CEP192 and CEP152 bind to Plk4 in a hierarchical, spatio-temporally ordered and competitive fashion (Kim, Park et al. 2013, Park, Park et al. 2014). CEP192 is critical for Plk4 recruitment to centrosomes, while co-depletion of CEP192 and CEP152 results in complete loss of Plk4 from the centrosome, and thereby impairs centriole duplication (Kim, Park et al. 2013, Sonnen, Gabryjonczyk et al. 2013). Impairment of either the CEP192- or CEP152-dependent Plk4 interaction is sufficient to induce a defect in Sas-6 recruitment to the procentriole assembly site (Kim, Park et al. 2013). Plk4 has also been shown to phosphorylate Ser151 of FBXW5 reducing the activity of the SCF-FBXW5
E3-ubiquitin ligase during the G1/S transition, suppressing its ability to ubiquitinylate Sas-6 (Puklowski, Homsi et al. 2011). In addition, recent work shows that phosphorylation of STIL by Plk4 facilitates the Sas-6-STIL interaction and centriolar loading of Sas-6, a major component of the centriolar cartwheel (Dzhindzhev, Tzolovsky et al. 2014, Ohta, Ashikawa et al. 2014). Continued Plk4 activity is required for STIL localization to the centrosome, and primes the binding of STIL to Sas-6 (Moyer, Clutario et al. 2015). In turn, STIL regulates the centriolar distribution of Plk4 and activity and may in this way restrict the occurrence of procentriole seeding to one site on each mother centriole (Ohta, Ashikawa et al. 2014, Moyer, Clutario et al. 2015).

1.1.5 Plk4 Interactions and Substrates

For over a decade, it has been recognized that Plk4 localizes primarily to centrosomes and is essential for centriole duplication, while its role in cytokinesis was described more recently and remains controversial (Bettencourt-Dias, Rodrigues-Martins et al. 2005, Rosario, Ko et al. 2010, Holland, Fachinetti et al. 2012). It is a serine-threonine kinase for which few bona fide functional substrates had been identified until recently, although the field has changed dramatically over the past five to six years. In 2010, centrosome fractions and Xenopus egg extracts were analyzed for interacting proteins using a proteomic approach, with CEP152/Asterless being identified as a key Plk4 centrosomal scaffold (Cizmecioglu, Arnold et al. 2010, Hatch, Kulukian et al. 2010, Sonnen, Gabryjonczyk et al. 2013). This was followed by identification of CEP192 as a further scaffolding protein, which cooperates with CEP152 in a hierarchical fashion in the centriolar recruitment of Plk4 and centriole duplication (Kim, Park et al. 2013, Sonnen, Gabryjonczyk et al. 2013, Park, Park et al. 2014). Most recently, STIL/Ana2 was identified as a key Plk4 substrate, for the first time linking Plk4 catalytic kinase activity to centriole duplication (Dzhindzhev,
Tzolovsky et al. 2014, Ohta, Ashikawa et al. 2014). Other interactors reported to be involved in centriolar duplication include: CDK11p58, required for CEP192/Plk4 recruitment to the centrosome and centriole maturation (Franck, Montembault et al. 2011); GCP6, a core component of the γ-tubulin ring complex which physically interacts with and is phosphorylated by Plk4 (Bahtz, Seidler et al. 2012); FBXW5 (Puklowski, Homsi et al. 2011); CEP78 which is required for Plk4-induced centriole overduplication (Brunk, Zhu et al. 2016); and the heretofore-unknown protein with unknown function FAM46C (unpublished). FAM46C is a conserved protein identified in two separate yeast two-hybrid screens as a potential Plk4 interactor (Rual, Venkatesan et al. 2005, Murali, Pacifico et al. 2011). FAM46C consists of 391 amino acid residues, and is found on human chromosome 1 at the locus 1p12. FAM46C contains one predicted domain of unknown function, DUF1693, and shares considerable amino acid identity with three other HsFAM46 proteins (A, B, D). FAM46C has not been crystallized and its tertiary structure has not yet been determined. Little is known about the function of FAM46C, although it has been suggested that FAM46C may be functionally involved with the Type 1 interferon response (Schoggins, Wilson et al. 2011). FAM46C is recurrently mutated and inactivated in multiple myeloma in a pattern typical of a tumour suppressor gene, and associates with a hyperdiploid karyotype subgroup (Boyd, Ross et al. 2011, Chapman, Lawrence et al. 2011, Bolli, Avet-Loiseau et al. 2014). In addition, deletion and/or mutation of FAM46C in one study was associated with impaired overall survival in myeloma patients (Boyd, Ross et al. 2011).

Plk4 has been shown to bind and be subject to FBXW1/β-TrCP-dependent proteasomal degradation subsequent to autophosphorylation within the phosphodegron motif (Guderian, Westendorf et al. 2010, Holland, Lan et al. 2010), with tight regulation of PLK4 by SKP1–Cullin 1 (CUL1)–F-box (SCF) E3 ubiquitin ligase activity to prevent the synthesis of extra daughter
CAND1 is a centrosomal protein that inhibits the assembly of multi-subunit E3 ubiquitin ligase complexes by binding with inactive CUL1, competing with SKP1, and generally suppressing SCF ubiquitin ligase activity. CAND1 augments the stability of Plk4 and promotes Plk4 induced centriole overduplication (Korzeniewski, Hohenfellner et al. 2012). In prostate cancer, CAND1 expression is disrupted at both the mRNA and protein levels (Korzeniewski, Hohenfellner et al. 2012). In Drosophila S2 cells, PP2A (Protein Phosphatase 2A(Twins)) counteracts fly Plk4/SAK autophosphorylation, thus stabilizing SAK and promoting centriole duplication (Brownlee, Klebba et al. 2011). Plk4 has also been shown to bind and phosphorylate the RhoA GEF Ect2, and regulate Ect2 localization to the spindle midbody and resultant RhoA activation at cytokinesis (Rosario, Ko et al. 2010). Hand1 is a nucleolar substrate of Plk4 involved in trophoblast differentiation where phosphorylation by Plk4 disrupts the interaction between HAND1 and MDFIC, activating HAND1 (Martindill, Risebro et al. 2007). Finally, Cdc25c and Chk2 have been shown to be phosphorylated by Plk4 in in vitro kinase assays, although the functional consequences in cells are not clear (Bonni, Ganuelas et al. 2008, Petrinac, Ganuelas et al. 2009).

Regulation of Plk4 by p53, NFκB and PTEN has also been described. When considering the relationship between Plk4 and p53, initial studies showed that Plk4 interacts with p53 in NIH3T3 cells in a co-immunoprecipitation assay (Swallow, Ko et al. 2005). In addition, Plk4 expression was shown to be transcriptionally repressed by p53 via the recruitment of histone deacetylase (HDAC) repressors, where SAK/Plk4 overexpression attenuated p53-induced apoptosis (Li, Tan et al. 2005). Microarray analysis also revealed significant differences between Plk4 wild-type and heterozygous murine embryonic fibroblasts (MEFs), where expression levels of genes involved in p53 dependent pathways were altered (Morettin, Ward et al. 2009). Morettin et al.
proposed that increased p53 levels in cells were invoked as a result of Plk4 haploinsufficiency (Morettin, Ward et al. 2009), while Ko et al. found impaired p53 activation in Plk4+/− regenerating mouse liver (Ko, Rosario et al. 2005). Interestingly, in normal mouse liver, ChIP analysis showed binding of p53 to Plk4, and activation of Plk4 expression (Kurinna, Stratton et al. 2013), although direct binding of Plk4 to p53 remains controversial. In 2012 Holland et al. showed that preventing Plk4 autodestruction through expression of Plk4+/AA or Plk4+/Δ24 conditional alleles caused centrosome amplification, resulting in p53 stabilization and loss of cell proliferation; p53 suppression using shRNA or inhibition with the SV40 large T-antigen subsequently allowed growth of cells carrying amplified centrosomes (Holland, Fachinetti et al. 2012). A similar block of proliferation was seen with increased levels of the centrosomal structural component Sas-6 in cells (Holland, Fachinetti et al. 2012). Overall, p53 transcriptionally represses Plk4 expression and, in cells, Plk4 haploinsufficiency and Plk4 stabilization lead to increased p53 stabilization/activity to affect cell proliferation.

In keeping with previous studies showing transcriptional repression of Plk4 by p53 (Li, Tan et al. 2005, Fischer, Quaas et al. 2014), Nakamura et al. showed that genotoxic stresses, such as etoposide treatment or ultraviolet irradiation, gradually downregulated Plk4 protein expression in a p53-dependent manner (Nakamura, Saito et al. 2013). Further elucidating the relationship between stress and Plk4, they showed that in response to stress stimuli, Plk4 is directly phosphorylated at T170 in the kinase activation loop and activated by stress-activated protein kinase kinase kinases (SAPKKKs) (including TAK1, MEKK1, MLK3), where in addition to its role in centriole duplication, Plk4 also serves to suppress stress-induced apoptotic cell death. In cancer cells, high Plk4 levels could thus facilitate survival and proliferation despite environmental exposure to oxidative stress. Under stress conditions, this study also suggested
that the numeral integrity of centrosomes is preserved by synergistic actions of both stress-activated protein kinases (SAPKs; p38 and JNK), which are rapidly activated downstream of the SAPKKKs in stress to inhibit Plk4-mediated centriole overduplication although the precise mechanism remains unknown, and p53, which when stress continues is gradually activated to downregulate Plk4, promoting apoptosis and reducing the risk of Plk4-induced centrosome amplification (Nakamura, Saito et al. 2013).

The transcription factor NFκB also regulates Plk4 expression, with depletion of the NFκB subunits reducing Plk4 expression. NFκB binds the Plk4 promoter to regulate its expression, and long-term depletion of NFκB2 results in defects in centrosome structure (Ledoux, Sellier et al. 2013). This pathway further elucidated the transcriptional regulation of Plk4 in response to stimuli such as stress and infection. Plk4 expression may also be regulated by epigenetic mechanisms in response to hypoxia or reactive oxygen species, with promoter hypermethylation and subsequent decreased expression seen in the presence of functional p53 (Ward and Hudson 2014). Further, promoter methylation of Plk4 CpG islands and subsequent downregulation of expression correlated with the development of hepatocellular carcinoma in Plk4+/− mice (Ward, Morettin et al. 2011). The effect of hypoxic culture conditions on Plk4 expression was also observed by our group, where hypoxia increased Plk4 expression in MEFs, particularly in Plk4+/− MEFs (Rosario, unpublished).

Interestingly, the development of a specific and reversible inhibitor of Plk4, centrinone and centrinone B, showed that Plk4 inhibition and subsequent centrosome loss triggered a p53-dependent arrest in normal cells, that was independent of DNA damage, stress, Hippo signaling, extended mitotic duration, or segregation errors. Consistent with this, immunoblot analysis
showed increased levels of p53 and its downstream effector p21 after centrosome depletion. However, cancer cells, a large proportion of which had mutations in or suppress expression of p53, continued to proliferate indefinitely albeit at a lower basal rate (Wong, Anzola et al. 2015). In keeping with this, Lambrus et al. used an auxin-inducible degradation system to deplete Plk4, which led to a failure of centriole duplication, where this failure of centriole duplication itself increased p53 levels that produced an irreversible cell cycle arrest that was not a result of a prolonged mitosis, chromosome segregation errors, or cytokinesis failure. Again, depleting p53 allowed cells that fail centriole duplication to proliferate indefinitely, and although acentriolar cell divisions occurred they exhibited an increased mitotic duration and were associated with a higher frequency of chromosome segregation errors and cytokinesis failure leading to a subpopulation of acentriolar cells containing tetraploid DNA content with an observed reduced growth rate and increased doubling time. Restoring endogenous Plk4 levels in acentriolar cells promoted the penetrant formation of de novo centrioles, although a single centriole was shown to suppress de novo centriole assembly (Lambrus, Uetake et al. 2015). Recent studies have now identified the USP28-53BP1-p53-p21 signaling pathway that arrests the cell cycle following Plk4 inhibition and centrosome loss (Fong, Mazo et al. 2016, Lambrus, Daggubati et al. 2016, Meitinger, Anzola et al. 2016).

The role of p53 in Plk4 overexpression and centrosome amplification was further demonstrated in vivo in mouse models, where p53-dependent pathways invariably prevented the proliferation of cells with supernumerary centrosomes due to Plk4 overexpression. Concomitant Plk4 overexpression and p53 deletion rescued the apoptosis in cells with extra centrosomes, promoting genomic instability and aneuploidy (Marthiens, Rujano et al. 2013, Coelho, Bury et al. 2015, Kulukian, Holland et al. 2015, Vitre, Holland et al. 2015, Sercin, Larsimont et al.)
Nevertheless, while Vitre et al. did not find that Plk4-driven centrosome amplification promoted tumourigenesis, even in p53 null or heterozygous mice, this was in contrast to studies by Coelho et al. (Coelho, Bury et al. 2015) where in the p53 null background, there was accelerated tumour formation, namely lymphomas and sarcomas in the Plk4 overexpressing mice, and Sercin et al. (Sercin, Larsimont et al. 2016) showing that Plk4 overexpression in mice promoted tumourigenesis in p53 deficient epidermis. Interestingly, the development of lymphomas and sarcomas was accelerated in Plk4^{+/−} p53^{−/−} compared with Plk4^{+/+} p53^{−/−} mice (Rosario, Ko et al. 2010), suggesting that tumour induction through Plk4 haploinsufficiency is also facilitated by p53 deficiency. Overall, both centrosome amplification (Holland, Fachinetti et al. 2012) and centrosome loss (Lambrus, Uetake et al. 2015, Wong, Anzola et al. 2015) lead to a p53-dependent cell cycle arrest in cells, and p53 prevents centrosome amplification in many tissue types in mice (Coelho, Bury et al. 2015, Kulukian, Holland et al. 2015, Vitre, Holland et al. 2015, Sercin, Larsimont et al. 2016).

Functional interaction has also been reported between Plk4 and the tumour suppressor PTEN in breast cancer cells, suggesting that PTEN-deficient breast tumour cells are dependent on Plk4 for survival (Brough, Frankum et al. 2011). When evaluating breast cancer cell lines for response to the Plk4/AuroraB kinase inhibitor, CFI-400945, a correlation between induction of cell death and PTEN deficiency was noted along with increased \textit{in vivo} activity in PTEN null relative to PTEN wild-type tumour models, linking response to CFI-400945 treatment and PTEN status (Mason, Lin et al. 2014).

In summary, accumulating evidence suggests that Plk4 also functions outside of the centrosome and is involved in controlling several essential cellular functions including cytokinesis, apoptosis, gene expression and cell motility, which will be discussed further
(Morettin, Ward et al. 2009, Rosario, Ko et al. 2010, Nakamura, Saito et al. 2013, Rosario, Kazazian et al. 2014). One of my aims is to identify and further describe additional Plk4 functional interactors, primarily in the promotion of cell motility and invasion. Abnormalities in the Plk4 network could predispose patients to carcinogenesis, as has been shown for Plk4 itself in hepatocellular cancer.

1.2 Plks in carcinogenesis and cancer progression

1.2.1 Plk1

2002, Mito, Kashima et al. 2005, Weichert, Kristiansen et al. 2005), and some reports have specifically identified high Plk1 expression as a marker of metastatic risk (Ahr, Karn et al. 2002, Kneisel, Strebhardt et al. 2002). It has therefore been suggested that elevated Plk1 activity may drive tumour progression, though direct evidence for this is not as abundant. Inhibition of Plk1 does indeed impair cancer cell growth and reduce viability: siRNA or small molecule inhibitors induce prolonged mitotic arrest and subsequent apoptosis in cancer cells (Spankuch-Schmitt, Bereiter-Hahn et al. 2002, Liu and Erikson 2003, Guan, Tapang et al. 2005, Gumireddy, Reddy et al. 2005, Reagan-Shaw and Ahmad 2005). In preclinical studies, this has translated to tumour shrinkage in mice (Spankuch-Schmitt, Wolf et al. 2002, Guan, Tapang et al. 2005, Gumireddy, Reddy et al. 2005, Steegmaier, Hoffmann et al. 2007). Importantly, non-transformed cells are less dependent on Plk1 expression and therefore less susceptible to its inhibition (Cogswell, Brown et al. 2000, Liu, Lei et al. 2006). Moreover, constitutive expression of Plk1 in NIH 3T3 fibroblasts transforms the cells, enabling tumour growth when injected in nude mice (Smith, Wilson et al. 1997). Thus, significant evidence has accumulated to support Plk1 as a valid drug target, and multiple clinical trials have tested the safety and efficacy of this strategy in patients. In particular, volasertib, an ATP-competitive inhibitor of Plk1, is currently in Phase III trials due to encouraging antitumour activity in solid tumours and was granted Breakthrough Therapy designation by the FDA for significant benefit in treating patients with AML in combination with low-dose cytarabine (Gjertsen and Schoffski 2015).

Despite evidence suggesting that Plk1 may promote tumour progression, deficiency phenotypes in mice point to a potential tumour suppressive function. Complete absence of Plk1 is embryonic lethal, while mice heterozygous for Plk1 are viable, develop normally, and are fertile (Lu, Wood et al. 2008). With advancing age, however, Plk1<sup>−/−</sup> mice develop tumours,
including lymphomas, lung adenocarcinomas, squamous cell carcinoma and ovarian sarcoma, at an increased incidence compared to their wildtype littermates, implicating Plk1 as a haploinsufficient tumour suppressor (Lu, Wood et al. 2008). By contrast, Raab et al. (Raab, Kappel et al. 2011) reported that mice with conditional knockdown of Plk1 with siRNA did not develop tumors at a higher rate than controls, even after 30 months of doxycycline treatment; using this targeting strategy, the only apparent sequela was a moderate reduction in mitotic index in ovarian follicles and in mucosal folds of the large intestine.

1.2.2 Plk2, Plk3 and Plk5

Decreased Plk2 expression, often through epigenetic gene silencing, has been described in various tumors including B cell malignancies (Smith, Syed et al. 2006, Syed, Smith et al. 2006, Li, Lu et al. 2008, Benetatos, Dasoula et al. 2011), multiple myeloma (Hatzimichael, Dasoula et al. 2010) and hepatocellular carcinoma (Pellegrino, Calvisi et al. 2010). This, together with its role in initiating the DNA damage response, has prompted the suggestion that it may function as a tumour suppressor. However, neither heterozygous nor null mice were reported to develop tumours at a higher rate than their wildtype counterparts (Table 1). Interestingly, low expression of Plk2 has been linked to decreased sensitivity to chemotherapy in ovarian cancer (Ju, Yoo et al. 2009), multiple myeloma (Hatzimichael, Dasoula et al. 2010) and leukemia (de Viron, Knoops et al. 2009), and it has been suggested that Plk2 methylation status and/or expression may be used as a marker to predict chemosensitivity in cancer patients (Syed, Coley et al. 2011). There has been one report of overexpression of Plk2 in bladder carcinoma (Tan, Chen et al. 2010).

Decreased Plk3 expression has been found in hepatocellular carcinoma (Pellegrino, Calvisi et al. 2010), head and neck (Dai, Li et al. 2000), non-small cell lung (Li, Ouyang et al. 1996),
uterne (Ando, Ozaki et al. 2004) and bladder (Ando, Ozaki et al. 2004) cancers. In one study, elderly mice lacking Plk3 developed liver, lung, uterus and kidney tumours at a higher frequency than wildtype mice (Yang, Bai et al. 2008), giving support to idea that Plk3 acts as a tumour suppressor. Using a somewhat different targeting strategy, Myer et al. (Myer, Robbins et al. 2011) found no increase in tumour formation in Plk3<sup>−/−</sup> mice. Despite some evidence for Plk3 as a tumour suppressor, a few studies have reported elevated Plk3 levels in some cancer types including cholangiocarcinoma, where it was correlated with prolonged patient survival, and breast (Weichert, Kristiansen et al. 2005) and ovarian carcinomas (Weichert, Denkert et al. 2004). Relatively little is known about the role of Plk5 in tumourigenesis. Given its restricted expression (brain only), Plk5 has been assessed in astrocytoma and glioblastoma multiforme, where its expression appears to be reduced (de Carcer, Escobar et al. 2011). In these tumours Plk5 is silenced epigenetically.

**1.2.3 Plk4**

Errors in cell division can lead to genomic instability and predisposition to carcinogenesis. Given the central role played by Plk4 in centriole duplication and perhaps also cytokinesis (Bettencourt-Dias, Rodrigues-Martins et al. 2005, Habedanck, Stierhof et al. 2005, Kleylein-Sohn, Westendorf et al. 2007, Rosario, Ko et al. 2010, Sillibourne, Tack et al. 2010), it is not surprising that dysregulation of Plk4 levels, whether up or down, leads to multipolar spindle formation, mitotic errors and chromosomal instability (Fujiwara, Bandi et al. 2005, Ko, Rosario et al. 2005, Lingle, Lukasiewicz et al. 2005, Basto, Brunk et al. 2008, Rosario, Ko et al. 2010, Marthiens, Rujano et al. 2013). Impairment of Plk4-dependent mitotic fidelity can facilitate
cancer development, as manifest in the promotion of tumourigenesis by insufficient levels of Plk4 (Ko, Rosario et al. 2005, Rosario, Ko et al. 2010). Haploid levels of Plk4 result in immortalization of MEFs in culture, with the consistent development of a near-tetraploid karyotype, leading to aneuploidy and the ability to generate tumours in vivo following subcutaneous injection in NOD SCID mice. Plk4+/− mice develop normally but have a 50% incidence of spontaneous tumour formation by the age of 24 months, the majority being multifocal hepatocellular carcinomas and others including primary lung adenocarcinomas and sarcomas; these were all markedly increased in incidence compared to age-matched Plk4+/+ mice (Ko, Rosario et al. 2005). In addition, after partial hepatectomy, 100% of Plk4+/− mice develop diffuse high-grade hepatic dysplasia within 6-9 months, further indicating that Plk4 is haploinsufficient for tumor suppression (Ko, Rosario et al. 2005). Human Plk4 maps to chromosome 4q28, a region that is frequently lost in hepatomas (Hammond, Jeffers et al. 1999, Hudson, Chen et al. 2000). Further, analysis of human hepatocellular cancer specimens shows a loss-of-heterozygosity (LOH) at the Plk4 locus in approximately 50% of patients, with an accompanying decrease in Plk4 expression in the same patients’ tumours, as assessed by real-time RT-PCR (Rosario, Ko et al. 2010). In hepatomas with retention of heterozygosity at the Plk4 locus, Plk4 expression was increased on average 3-fold in tumour compared to adjacent normal liver (Rosario, Ko et al. 2010). In keeping with the effects of progressive chromosomal instability when levels of Plk4 are insufficient, reduced Plk4 expression has also been associated with worse survival in hepatoma patients (Liu, Zhang et al. 2012). This was similarly shown in another study, where decreased Plk4 mRNA and protein expression were seen in hepatocellular carcinoma, with lower levels of Plk4 in hepatomas with worse survival and Plk4 downregulation associated with loss of heterozygosity (Pellegrino, Calvisi et al. 2010). As an additional
mechanism, Ward and colleagues (Ward, Morettin et al. 2011) have described epigenetic modification with promoter hypermethylation, and subsequent downregulation of expression in Plk4, in Plk4 heterozygous mice that can contribute to the development of HCC. In hematologic malignancies, increased Plk4 promoter hypermethylation was identified in lymphoma and myelodysplastic syndrome (MDS)/leukemia bone marrow aspirates in comparison to normal (Ward, Sivakumar et al. 2015), and Plk4 gene expression down-regulation along with centrosome amplification was also previously described in multiple myeloma (Dementyeva, Kryukov et al. 2013). Preliminary evidence also suggests that chronic Hepatitis B virus (HBV) infection is associated with reduced Plk4 expression, indicating that deficient Plk4 kinase activity may play a mechanistic role in HBV-induced hepatocellular carcinogenesis (Rosario 2011). The role of Plk4 in viral carcinogenesis is further suggested by the dysregulation of Plk4 mRNA expression and centriole duplication in human papillomavirus type 16 (HPV-16) E7 oncoprotein-expressing keratinocytes (Korzeniewski, Treat et al. 2011).

On the other hand, emerging evidence suggests that increased Plk4 levels in established malignancy may promote tumour progression (Rosario, Kazazian et al. 2014). While recurrent mutations in Plk4 in human cancers are rare, high levels of Plk4 expression have been reported in many solid tumour types, including breast and colorectal cancers, glioblastoma, and bladder cancer (Macmillan, Hudson et al. 2001, Mason 2011, Mason, Lin et al. 2014). In breast cancer specifically, Plk4 has been identified as an element of a “stem cell like” expression signature that is associated with not only resistance to therapy but also death from metastases (Glinsky 2006, Finetti, Cervera et al. 2008, Agarwal, Gonzalez-Angulo et al. 2009, Mason, Lin et al. 2014), indicating that Plk4 expression level may not merely reflect proliferative status but may facilitate tumour progression. In keeping with the latter hypothesis, Plk4 has been shown to increase
cellular invasiveness *in vitro* (Rosario, Kazazian et al. 2015), although some groups attribute this solely to the action of supernumerary centrioles (Godinho, Picone et al. 2014). Our group has recently shown that Plk4 stimulates cell motility *in vitro* (Rosario, Kazazian et al. 2014). Following the identification of a gene expression pattern predictive of reduced motility in Plk4+/− MEFs, we showed in functional assays that Plk4 promotes cell spreading, migration and invasion *in vitro*. Cytoskeletal reorganization was impaired in Plk4+/− MEFs that had been stimulated to migrate. We also identified a novel localization of Plk4 to the protrusions of motile cells (Rosario, Kazazian et al. 2014). Godhino et al. (Godinho, Picone et al. 2014) used a 3-D culture system to suggest that centrosome amplification triggers mammary epithelial cell invasion, whereby increased centrosomal microtubule nucleation increases Rac1 activity and disrupts normal cell–cell adhesion to promote invasion (Godinho, Picone et al. 2014). Pellman’s group ascribed the increased invasiveness solely to centrosome amplification. Denu et al. (Denu, Zasadil et al. 2016) showed that in breast cancer, centrosome amplification is associated with reduced overall survival and recurrence-free survival, and correlates strongly with high-risk subtypes (triple negative and HER2-amplified) and higher stage and grade. In this study, a strong correlation between centrosome amplification and chromosomal instability was observed in breast cancers, and it was proposed that at least 15% of cases of centrosome amplification in human breast cancer arose by a doubling event, such as cytokinesis failure or cell-cell fusion. Interestingly, centrioles were absent from a sizeable proportion of centrosomes in the tumour samples, thought to result from pericentriolar material fragmentation. In cells, induction of centrosome amplification using Plk4 induced high-grade features with cells that were more dedifferentiated, supporting the idea that centrosome amplification intrinsically causes high-grade tumours (Denu, Zasadil et al. 2016).
Initial studies to explore SAK/Plk4 overexpression and tumourigenesis in *Drosophila* showed that elevated expression of Plk4 and resultant centrosome amplification in larval brain cells, when transplanted into the abdomens of wildtype adult flies, can generate metastatic tumours to the eye (Basto, Brunk et al. 2008). To study the effects of elevating Plk4 expression in the murine brain, Marthiens et al. (Marthiens, Rujano et al. 2013) generated transgenic mice with tissue-specific conditional overexpression of Plk4 in the embryonic central nervous system. This resulted in a dramatic reduction in brain size at birth, that is microcephaly, ascribed to poor clustering of centrosomes, multipolar spindles, chromosome segregation defects and consequent apoptosis, although there was no observed tumourigenesis even in a p53-depleted background (Marthiens, Rujano et al. 2013). Of note, Plk4 overexpressing pups had decreased survival and could not be followed to advanced age. Also, in human brain, mutations in Plk4 with reduction in centriole number result in microcephaly, primordial dwarfism and additional congenital anomalies, including retinopathy (Martin, Ahmad et al. 2014), further implying that aberrations in centriole biogenesis may significantly impair development of the mammalian brain.

While Basto et al. found that *Drosophila* lines overexpressing SAK with extra centrosomes show no dramatic increase in genetic instability, with extra centrosomes clustering together to form bipolar mitotic spindles, in the Plk4 overexpressing murine brain the neural stem cell population is vulnerable to aberrant spindle formation with resultant aneuploidy (Basto, Brunk et al. 2008, Marthiens, Rujano et al. 2013). In a recent study by Lambrus and colleagues (Lambrus, Uetake et al. 2015), an auxin-inducible degradation system was utilized to deplete Plk4 protein in RPE-1 cells, leading to a failure in centriole duplication and subsequent irreversible cell cycle arrest, in a p53 dependent manner. While failure of centriole duplication increased p53 levels, eliciting a cell cycle arrest, depleting p53 allowed cells that fail centriole duplication to
proliferate indefinitely; the latter was associated with a higher frequency of chromosome segregation errors and cytokinesis failure resulting in a population of cells with a tetraploid DNA content, revealing a p53-dependent surveillance mechanism that protects against genome instability by preventing cell growth after centriole duplication failure (Lambrus, Uetake et al. 2015). Nevertheless, the nature of the relationship between Plk4 related centriole gain or loss and a p53-dependent response in cells remains unclear, as DNA damage, stress, chromosome missegregation, prolonged mitosis, Hippo signaling and cytokinesis failure have not been shown to be responsible for triggering the p53-dependent cell arrest (Lambrus, Uetake et al. 2015), suggesting an interesting area of research.

In mammalian tissues, it is evident that despite the strong association between centrosome amplification and tumourigenesis, extra centrosomes negatively impact the fitness of mammalian cells and tissues (Marthiens, Rujano et al. 2013, Lambrus, Uetake et al. 2015, Vitre, Holland et al. 2015). To further study the relationship between centrosome amplification and tumourigenesis, Vitre et al. (Vitre, Holland et al. 2015) developed a mouse model in which widespread Plk4 overexpression was induced using a tamoxifen inducible Cre-recombinase event, where young mice were exposed to tamoxifen for 2 months. This allowed them to overcome the early embryonic lethality associated with Plk4 overexpression in the embryo. In mice with active p53, centrosome amplification was noted in the liver and skin, but there was no accelerated tumourigenesis or difference in overall survival when compared to control despite long-term follow-up. Even when one allele of p53 was inactivated or in p53 null mice, Plk4 overexpression did not effect tumour formation or influence tumour-free survival (Vitre, Holland et al. 2015). In addition, in mice with conditional Plk4 overexpression in the epidermis under control of the keratin 14 promoter (K14Cre), application of the chemical mutagen 7,12-
dimethylbenz(a)anthracene (DMBA) followed by multiple applications of the tumour-promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) to generate papillomas did not affect number of tumours or survival in these animals. Untreated animals also did not develop tumours (Kulukian, Holland et al. 2015, Vitre, Holland et al. 2015). Interestingly, in a comparable approach, Coehlo and colleagues similarly utilized a Cre-recombination approach to generate transgenic mice with doxycycline inducible expression of Plk4, here leading to hyperproliferation of pancreatic islet cells and epidermal hyperplasia, reflecting a shift in the balance between proliferation and differentiation. Notably, in the p53 null background, there was advanced tumour formation, namely lymphomas and sarcomas in the Plk4 overexpressing mice, differing from the results shown by Vitre et al. (Coelho, Bury et al. 2015, Vitre, Holland et al. 2015). In keeping with previous studies, behavioural defects were also evident suggestive of abnormalities in brain development (Coelho, Bury et al. 2015). Sercin and colleagues (Sercin, Larsimont et al. 2016) similarly developed a genetic mouse model conditionally overexpressing mCherry-Plk4 with K14Cre, allowing for K14Cre mediated recombination and overexpression of mCherry–Plk4 during mouse skin epidermis development. Using this approach, 90% of the cells expressing mCherry–Plk4 exhibited centrosome amplification leading to abnormal mitoses and aneuploidy. In the presence of p53, Plk4 overexpression was found to be highly deleterious for cell fitness and epidermal development, resulting in delayed epidermal stratification leading to skin barrier defects and neonatal lethality in about 50% of mice. In mice that survived postnatally, Plk4 overexpressing cells were eliminated by both p53-dependent and p53-independent apoptosis and Plk4 transgene expression was shut down. p53 deletion rescued the defects in skin differentiation and barrier formation mediated by Plk4 in the epidermis, with a higher percentage of mitotic cells and an increase in self-renewing divisions, although it did not prevent the
apoptosis of Plk4 overexpressing cells. Plk4 was transcriptionally shut down, centrosome amplification was suppressed irrespective of p53 expression, although p53 deletion combined with Plk4 overexpression resulted in the generation of aneuploidy and triggered the formation of squamous cell carcinomas with complete penetrance; while neither control nor Plk4-overexpressing mice developed spontaneous skin tumours, all Plk4 overexpressing/p53KO mice developed spontaneous skin tumours with a shorter latency and an increased number of tumours per mouse than p53KO alone (Sercin, Larsimont et al. 2016).

Overall, it is apparent that the relationship between Plk4 and cancer is complex. In order to develop effective anti-Plk4 therapy and to select patients appropriately, greater understanding of the mechanisms of Plk4-related tumourigenesis and tumour progression is required, including the pathways/networks through which cancer cell invasion and metastasis are regulated.

1.3 Plk4 inhibitors

Mason and colleagues (Laufer, Forrest et al. 2013, Mason, Lin et al. 2014, Sampson, Liu et al. 2014) first described the development of an orally active Plk4 inhibitor, CFI-400945, a potent ATP-competitive inhibitor which has an intriguing bimodal effect on centriole number; complete inhibition of Plk4 with CFI-400945 leads to a failure of centriole duplication, while lower doses and partial inhibition interestingly result in an increase in centriole number (Mason, Lin et al. 2014), akin to the heterozygote phenotype observed in mice (Ko, Rosario et al. 2005).

Regardless of partial or complete Plk4 inhibition, CFI-400945 leads to dysregulated centriole duplication, defects in cytokinesis, accumulation of polyploid cells and eventually cell cycle arrest or death (Mason, Lin et al. 2014). Mason and colleagues (Mason, Lin et al. 2014) reported that CFI-400945 did not have significant inhibitory activity against Plk’s 1-3, but did show
activity against several mitotic kinases both in vitro and in cells, including Aurora B kinase (AURKB), which could explain some of the observed effects of CFI-400945. In particular, the observed failure of cytokinesis and development of polyploidy with the accumulation of cells having ≥4N DNA content, was ascribed to Aurora B inhibition (Keen and Taylor 2009, Mason, Lin et al. 2014). Preclinical experiments in mice using breast cancer cell lines, xenografts derived from primary human breast cancers and xenografts derived from HCT116 and SW48 colorectal cancer cell lines showed that CFI-400945 is well-tolerated and has effective anti-tumour activity (Mason, Lin et al. 2014). CFI-400945 treatment resulted in increased in vitro breast cancer cell death and in vivo activity in xenografts in PTEN null compared to PTEN wild-type cell lines. And in fact, it has recently been reported that Plk4 is an essential gene in PTEN-deficient breast cancer cells, which are particularly sensitive to Plk4 depletion (Brough, Frankum et al. 2011). A Phase 1 clinical trial has been initiated; however, the in vivo consequences of a potential centrosomal amplification phenotype and the issue of uncontrolled dosing of Plk4 inhibitors are caveats to bear in mind as clinical testing progresses. In addition, the inhibition of Aurora B kinase by CFI-400945 complicates understanding of the Plk4-related mechanism of action.

More recently, Wong et al. (Wong, Anzola et al. 2015) described the development of a specific, reversible inhibitor of Plk4, centrinone or centrinone B, which causes centrosome depletion and coincident decrease in cell proliferation. While cancer cells were able to proliferate indefinitely after centrinone-induced centrosome loss, normal human cells arrested in G1 by a p53-dependent mechanism independent of DNA damage, stress response, Hippo signaling, extended mitotic duration, or segregation errors (Wong, Anzola et al. 2015). Centrinone now offers a valuable new tool to specifically study the effects of Plk4 on cancer progression, though
there is as yet no orally bioavailable formulation.

### 1.4 Cell Migration

Cell motility is a highly coordinated and complex process, and is required as the foundation for many other biological processes, such as embryogenesis, immune surveillance and wound healing/tissue repair. In turn, aberrant regulation of cell migration drives progression of many diseases, including cancer invasion and metastasis. Eukaryotic cells have three main components to the cytoskeleton: microfilaments (composed of actin), microtubules (tubulin) and intermediate filaments (multiple different proteins as building blocks), which provide structure and shape, and are required for the initiation and perpetuation of cell migration. Actin filaments are the most abundant and the dominant structural component of cell protrusions. This thesis will focus primarily on actin-based cell migration, where cell movement is driven by spatially- and temporally-regulated actin polymerization at the leading edge. Six highly conserved actin isoforms are expressed in vertebrates, including the three α-isoforms which are expressed primarily in skeletal, cardiac, and smooth muscles, and the β- and γ-isoforms which are ubiquitously expressed. Actin is a 43 kDa globular protein; actin monomers polymerize to form actin filaments (F-actin) at the cell leading edge through adenoside triphosphate (ATP) hydrolysis, although ATP hydrolysis is not required for nucleation (Martin, Welch et al. 2006). The filaments are structurally arranged as double helical polymers in a head-to-tail pattern, which give the filament molecular polarity with one end called the barbed (+) end and the other the pointed (-) end. The barbed (+) end is generally associated with a high concentration of F-actin-ATP and is favoured for growth, and is oriented outward with respect to the cell surface (Small, Isenberg et al. 1978, Symons and Mitchison 1991, Chan, Bailly et al. 2000). Favourable filament assembly is achieved through classes of proteins including formins and the spire.
proteins, which nucleate unbranched filaments (Baum and Kunda 2005, Kovar, Harris et al. 2006), and the actin-related protein (Arp)2/3 complex, which facilitates branched filament assembly (Figure 1.4a). This network of branched actin filaments pushes the cell membrane forward to protrude pseudopodia, where the Arp2/3 complex nucleates a new “daughter” filament from the side of an existing “mother” filament at a characteristic ~70°C angle, and is the primary nucleator of new actin filaments that advance the leading edge of migrating cells (Figure 1.4b; reviewed in (Pollard 2007); (Mullins, Heuser et al. 1998, Amann and Pollard 2001)).
Three main classes of actin nucleators have been identified that bypass the need for spontaneous actin nucleation to initiate filament assembly: the Arp2/3 complex, spire and formins. The Arp2/3 complex mimics an actin dimer/trimer and functions as a template to initiate formation of a new actin filament that branches from the existing mother filament at a characteristic 70° angle. The Spire proteins function as a scaffold for polymerization of unbranched actin filaments by mediating longitudinal association of four actin subunits through tandem G-actin-binding WH2 domains. Formins also promote the nucleation of unbranched filaments, where a dimer of formin-homology-2 (FH2) domains stabilizes an actin dimer or trimer to facilitate nucleation, remaining associated with the barbed or (+) end of actin filaments. The Arp2/3 complex is comprised of the actin-related-proteins (Arp)2 and 3, as well as 5 subunits ArpC1-C5. It begins in an inactive open configuration. Upon binding of NPFs and phosphorylation, it undergoes a conformational change that primes the complex for activation with binding of the WCA-ATP-actin-Arp2/3 complex to the mother filament. ATP is hydrolyzed on Arp2, with nucleation of the daughter filament and formation of branched actin filaments. NPF; nucleation-promoting factor, CRIB; Cdc42/Rac Interactive Binding motif, SHD; Scar homology domain, W or WH2; WASP homology 2, C; central or connecting, A; acidic tail, Pro-rich; proline rich region.
1.4.1 The Arp2/3 complex

The Arp2/3 complex has a variety of cellular functions, including lamellipodial protrusion, adhesion, podosome formation, phagocytosis, endocytosis, exocytosis, vesicle and organelle motility, and trafficking within and from the Golgi apparatus. It is comprised of seven subunits, including the two actin-related proteins, Arp2 and Arp3, and five subunits, ARPC1 (40-kDa), ARPC2 (34-kDa), ARPC3 (21-kDa), ARPC4 (20-kDa) and ARPC5 (16-kDa). The complex is the primary nucleator of new branched actin filaments, initiating new filament polymerization by binding to the side of a preexisting filament, where Arp2 and Arp3 function as a template (Mullins, Heuser et al. 1998, Amann and Pollard 2001, Amann and Pollard 2001). The subunits provide a framework for positioning the two Arp’s at the pointed end of the daughter filament (Robinson, Tubbedsky et al. 2001, Rouiller, Xu et al. 2008). The complex is stabilized in an inactive state, the two Arps being positioned too far apart to form the first two subunits of a new actin filament, and has been revealed to undergo large structural changes to template the nucleation of the daughter filament, particularly of the Arp2 and ARPC3 subunits (Robinson, Tubbedsky et al. 2001, Rodal, Sokolova et al. 2005, Rouiller, Xu et al. 2008, Narayanan, LeClaire et al. 2011). The Arp2/3 complex alone is a weak actin nucleator, and its activation is facilitated by binding of the mother filament (Higgs, Blanchoin et al. 1999), ATP binding (Goley, Rodenbusch et al. 2004, Martin, Xu et al. 2005), and interaction with nucleation promoting factors (NPFs). NPFs are divided into Class I NPFs, which include Wiskott-Aldrich syndrome protein (WASP), neural (N)-WASP and suppressor of cyclic AMP repressor (SCAR; also called WASP-family verprolin-homologous protein (WAVE)) (Machesky and Insall 1998, Machesky, Mullins et al. 1999, Rohatgi, Ma et al. 1999, Yarar, To et al. 1999), and Class II NPFs, the S. cerevisiae actin-binding protein-1 (Abp1) (Goode, Rodal et al. 2001), Pan1
(Duncan, Cope et al. 2001) and cortactin (Weed, Karginov et al. 2000, Uruno, Liu et al. 2001, Weaver, Karginov et al. 2001). Class II NPFs are much less potent activators of the Arp2/3 complex, and their mechanism of Arp2/3 activation is not well understood. In 1999 several groups (Machesky, Mullins et al. 1999, Rohatgi, Ma et al. 1999, Winter, Lechler et al. 1999, Yarar, To et al. 1999) identified the WASP/Scar family proteins as the predominant activators of the Arp2/3 complex regulating nucleation activity, recognizing the link between many cell surface receptors and actin assembly. These proteins have a common domain structure, containing the carboxyl terminal WASP-Homology 2 (WH2 or W) motifs often occurring in tandem repeats for interaction with actin monomers, a central or connecting (C) motif, and the acidic tail (A). This WCA region constitutes the shortest polypeptide required for activation of nucleation with the Arp2/3 complex, where the C and A motifs interact with various subunits of the Arp2/3 complex, stabilizing its active conformation (Machesky and Insall 1998, Machesky, Mullins et al. 1999, Mullins 2000). The amino terminal regions are more divergent: WASP and N-WASP have a proline-rich domain containing a Cdc42/Rac interactive binding motif (CRIB), while Scar/WAVE, which is an effector of Rac, does not have a CRIB motif but has a Scar homology domain. Thus, activation of class I NPFs is primarily regulated by the activity of the small Rho-family GTPases Cdc42 and Rac1. Other Class I NPFs include fungal Myosin-I (Lee, Bezanilla et al. 2000, Lechler, Jonsdottir et al. 2001), metazoan CARMIL (capping protein ARP2/3 and myosin-I linker) (Jung, Remmert et al. 2001) and pathogen proteins ActA and RickA (Welch, Rosenblatt et al. 1998, Gouin, Egile et al. 2004).

Until recently the Arp2/3 complex was thought to be primarily regulated by WASP proteins. However, recent evidence indicates that members of the complex are also phosphorylated by serine-threonine kinases. The MAPK-activated protein kinase 2 phosphorylates Ser77 of the
ARPC5 subunit (Singh, Powell et al. 2003), and the p21-activated kinase (PAK) phosphorylates Thr21 of the ARPC1 subunit leading to motility in mammalian cells (Vadlamudi, Li et al. 2004). In addition, LeClaire et al. (LeClaire, Baumgartner et al. 2008) showed that phosphorylation on threonine and tyrosine residues of the Arp2 subunit is functionally necessary to promote the actin nucleating activity of the Arp2/3 complex. Using mass spectrometry and mutagenesis studies, phosphorylation of Arp2 at T237, T238 and Y202 was identified, and was shown to be necessary for activation of the Arp2/3 complex in pyrene-labeled actin assays in the presence of NPFs. The authors suggest the Arp2/3 complex is a “coincidence detector” requiring both NPFs and phosphorylation of Arp2 to increase actin nucleation activity. Interestingly, coincidence detection appears to be a common regulatory mechanism for actin-binding proteins, including cofilin and the NPFs (Rohatgi, Nollau et al. 2001, Frantz, Barreiro et al. 2008, Rivera, Vasilescu et al. 2009). For example, localized N-WASP-mediated actin polymerization in cells can require coincident binding of Nck and phosphatidylinositol 4,5 bisphosphate (PI(4,5)P(2)). (Rivera, Vasilescu et al. 2009). In addition, expression of a phosphorylation deficient Arp2 mutant with alanine substitutions at T237/238 and Y202 in Drosophila melanogaster Schneider S2 cells did not restore lamellipodia in cells treated with Arp2 siRNA, indicating that phosphorylation of Arp2 is functionally relevant for lamellipodia formation and membrane protrusion (LeClaire, Baumgartner et al. 2008). Narayanan et al. (Narayanan, LeClaire et al. 2011) then used computational simulations to show that phosphorylation of Arp2 at T237 and T238, which are near the interface of Arp3, ARPC2, and ARPC4 at the core of the complex, results in conformational changes that are permissive for reorientation of Arp2 relative to Arp3, allowing the complex to change toward a short-pitch dimer orientation required for activity. They therefore proposed a model in which phosphorylation destabilizes the inactive state, allowing
Arp2 to reorient itself into a state that is permissive for full activation by NPFs (Narayanan, LeClaire et al. 2011). More recently, this group went on to identify the first kinase shown to phosphorylate and increase the activity of the Arp2/3 complex, the Nck-interacting kinase (NIK), a Ste20/MAP4K4 serine/threonine kinase (LeClaire, Rana et al. 2015). NIK was shown to directly bind and phosphorylate Arp2, increasing actin nucleating activity of the Arp2/3 complex and promoting plasma membrane protrusion in response to epidermal growth factor (LeClaire, Rana et al. 2015).

NIK binds the receptor tyrosine kinase adaptor protein Nck, which is ubiquitously expressed and composed of one SH2 and three SH3 domains. Upon growth factor stimulation, Nck is recruited to transduce signals from receptor tyrosine kinases, including the epidermal growth factor (EGF) and platelet derived growth factor (PDGF) receptors. Several effector proteins have been shown to bind to Nck, including among others NIK, WASP, p21-activated protein kinase (PAK), the guanine nucleotide exchange factor Sos, and PRK2, to mediate its effects on the actin cytoskeleton and MAPK signaling pathways (Hu, Milfay et al. 1995, Rivero-Lezcano, Marcilla et al. 1995, Galisteo, Chernoff et al. 1996, Quilliam, Lambert et al. 1996, Su, Han et al. 1997). NIK activity has been shown to be necessary for epithelial cell membrane protrusion formation and invasion (Wright, Wang et al. 2003, Baumgartner, Sillman et al. 2006), and NIK−/− mice display early embryonic lethality with defects in mesoderm migration (Xue, Wang et al. 2001). NIK is the murine ortholog of human mitogen activated protein kinase kinase kinase (MAP4K4), which activates the e-Jun N-terminal kinase (JNK), a “stress-activated” MAPK, pathway through a mechanism dependent on MEKK1. MAP4K4 is also designated the HPK/GCK-like kinase (HGK) as it exhibits structural homology to hematopoietic progenitor kinase (HPK) and germinal centre kinase (GCK) (Yao, Zhou et al. 1999). It belongs to a group of
kinases related to the S. cerevisiae MAP4K, Ste20, and is further subcategorized to the GCK-IV subfamily. In addition to Arp2, NIK phosphorylates the ERM proteins (ezrin, radixin and moesin), which cross-link actin filaments to the plasma membrane to regulate membrane protrusion and cell-substrate adhesion (Baumgartner, Sillman et al. 2006), and the plasma membrane Na(+)–H(+) exchanger NHE1, a ubiquitously expressed plasma membrane ion exchanger that regulates intracellular pH and cell volume (Yan, Nehrke et al. 2001).

Although there are no published reports of Arp2/3-component knockout mammals, aberrant Arp2/3 complex function has been implicated in a number of disease conditions, where Arp2 is suggested to have a modulating role in cancer cell invasion. The Arp2/3 complex is localized to lamellipodia and pseudopodia at the leading edge (Kelleher, Atkinson et al. 1995, Machesky, Reeves et al. 1997, Welch, DePace et al. 1997), and lamellipodial protrusion is inhibited when Arp2/3 activity is reduced in various cell types using RNAi (Rogers, Wiedemann et al. 2003, Steffen, Faix et al. 2006) or inhibitory antibodies (Bailly, Ichetovkin et al. 2001). In addition, Arp2 localizes to podosomes and invadopodia, actin-rich structures with adhesive and protrusive activities that extend into and degrade the extracellular matrix, and siRNA treatment of Arp2/3 complex components (ARPC2; p34-Arc) inhibits invadopodia formation in invasive cancer cells (Yamaguchi, Lorenz et al. 2005). Further, gene expression analyses have revealed that genes involved in actin polymerization at the leading edge, including the WAVE-Arp2/3 complex, are upregulated in a subset of invasive breast cancer cells (Wang, Goswami et al. 2004). Immunohistochemical analysis has also identified Arp2 as overexpressed in lung and colorectal cancers, and in lung cancer Arp2 is suggested to be involved in aggressive tumour behaviours (Otsubo, Iwaya et al. 2004, Semba, Iwaya et al. 2006). The Arp2/3 complex is therefore likely to be vital in the process of tumour-cell invasion and metastasis.
1.4.2 Rho GTPases and the molecular mechanisms of cell migration

The regulation of actin dynamics, adhesion organization and the formation of lamellipodia and filopodia primarily involves signaling from the small Rho family guanosine triphosphate (GTP)-binding proteins (GTPases). Rho GTPases belong to the Ras family of GTPases, and act as molecular switches that cycle between GTP-bound (active) and GDP-bound (inactive) forms (Figure 1.5a). In mammals the Rho GTPase family consists of 22 members, subdivided into the Rac subfamily (Rac1, Rac2, Rac3, and RhoG), Cdc42 subfamily (Cdc42, TC10, TCL, Chip, and Wrch-1), RhoA subfamily (RhoA, RhoB, and RhoC) and others (RhoE/Rnd3, RhoH/TTF, Rif, RhoBTB1, RhoBTB2, Miro-1, Miro-2, RhoD, Rnd1, and Rnd2) (Ridley 2006). The best-characterized molecules are RhoA, which controls contractile actomyosin filaments (stress fibers) and focal adhesion formation, Rac1 for lamellipodia and Cdc42, which regulates filopodium formation. Cdc42 and Rac1 are critically involved in actin filament formation by regulating WASP family members, which in turn stimulate the actin-nucleating Arp2/3 complex (reviewed in (Higgs and Pollard 2001)). In the classic model of Rho GTPase dependent cell migration, Rac1 and Cdc42 are described to be active at the leading edge in order to promote protrusion formation, while RhoA is active in the cell body and rear in order to provide the actomyosin-mediated force needed for rear retraction. Rho GTPases also regulate many other signal transduction pathways, such as regulation of cell polarity, gene transcription, G1 cell cycle progression, assembly of the actomyosin contractile ring in mitosis, microtubule dynamics, adhesion, vesicular transport pathways and enzymatic activities including NADPH oxidation in phagocytes. Here I will discuss mainly the role of the Rho GTPases in actin dynamics.

Rho GTPases bind GDP and GTP with high affinity, where binding of GTP results in a
conformational change that activates downstream target effectors such as actin nucleation promoting molecules, adaptors and kinases to mediate their cellular functions (Figure 1.5a). The exchange of GDP to GTP, and thus the activation of Rho GTPases, is catalyzed by guanine nucleotide exchange factors (GEFs) that act downstream of numerous growth factor receptors, integrins, cytokine receptors, and cadherins. Rho GTPase inactivation results from the GTPase activity, where GTP is hydrolyzed to GDP, with upregulation of this intrinsic weak activity by GTPase activating proteins (GAPs). Further negative regulation is achieved through Rho guanine nucleotide dissociation inhibitors (GDIs), which bind Rho GTPases and sequester them in the cytosol and thus block their activation. Over 80 GEFs and 70 GAPs have been described, underlining the fact that Rho GTPase regulation is complex and while distinct functions are frequently classically ascribed to each of the three Rho GTPases, it has now been shown that there is an interdependence and complex interplay between each family member during cell migration (Machacek, Hodgson et al. 2009).
Figure 1.5: Rho family GTPases in actin dynamics.
a) Rho GTPases bind GDP or GTP. Guanine nucleotide exchange factors (GEFs) catalyse the release of GDP from the GTPase, allowing GTP to bind, while GTPase-activating proteins (GAPs) increase the intrinsic GTPase activity of the GTPase, resulting in hydrolysis of GTP to GDP and phosphate. GDP-bound Rho proteins can be sequestered by Rho guanine nucleotide dissociation inhibitors (GDIs). Active, GTP-bound RhoGTPases transduce signals through various effectors. 
b) The downstream targets of active Rho GTPases in actin dynamics include p21-activated kinase (PAK), Rho-associated coiled-coil kinase (ROCK) and nucleation promoting factors, such as mammalian Diaphanous formin (mDia), Wiskott-Aldrich syndrome protein (WASP) or Wiskott-Aldrich syndrome protein-family verprolin homologous protein (WAVE). PAK phosphorylates LIM-motif containing kinase (LIMK), which phosphorylates and inhibits coflin regulating actin-filament turnover. Rho GTPases also promotes myosin actin interactions; ROCK phosphorylates myosin II light chain (MLC), myosin light chain phosphatase (MLCP), and LIMK. Phosphorylation of MLC or MLCP affects the amount of phosphorylated myosin light chain, contributing to contractility.
1.4.2.1 RhoA

During cell migration, RhoA is involved in both actin polymerization and force generation, through binding of one of its two main effectors, mDia (mammalian homolog of Drosophila diaphanous), and activation of the kinase ROCK (Rho-associated coiled-coil forming kinase/Rho kinase/ROK) (reviewed in (Narumiya, Tanji et al. 2009). mDia is part of the formin family of proteins, which function to catalyze actin nucleation and polymerization to produce long, straight actin filaments. In turn, ROCK functions by phosphorylation of a variety of substrates, including the myosin-binding subunit of myosin light chain phosphatase, which ROCK inactivates by phosphorylation leading to activation of myosin II, as well as directly phosphorylating the myosin light chain (Kimura, Ito et al. 1996). These two actions of ROCK, as a consequence, increase the myosin light chain phosphorylation, stimulate cross-linking of actin by myosin and enhance actomyosin contractility. ROCK also functions to phosphorylate LIM-kinase, which subsequently is activated and phosphorylates the actin filament depolymerizing and disassembling protein, ADF/cofilin, resulting in its inactivation and serving to stabilize existing actin filaments (Maekawa, Ishizaki et al. 1999). These actions downstream of RhoA are conducive to the formation of actomyosin bundles in the cell, specifically stress fibers and the contractile ring, two typical actin cytoskeletal arrangements induced by Rho and composed of anti-parallel actin filaments cross-linked by myosin II. In migrating cells, Rho-ROCK signaling is also involved in tail retraction by negatively regulating integrin adhesions in the tail (Worthylake, Lemoine et al. 2001, Yoshinaga-Ohara, Takahashi et al. 2002). RhoA also antagonizes Rac1-mediated signaling through ROCK/contractility-dependent activation of the RacGAP ARHGAP22, a key mediator of amoeboid movement in melanoma cells (Sanz-Moreno, Gadea et al. 2008). In contrast to the Arp2/3 complex, formins nucleate the formation of
unbranched actin filaments via the formin homology domain 2 (FH2). RhoA activates mDia. In turn, mDia accelerates actin nucleation and elongation, where the FH2 domains self-associate in a dimer and nucleate filament assembly by interacting with the plus or barbed ends (fast-growing ends) of actin filaments. One formin of a dimer dissociates from the barbed end to processively add actin monomers to the barbed end, and the other remains bound to the barbed end, thereby protecting the rapidly elongating barbed ends from actin capping proteins which largely inhibit filament elongation (reviewed in (Zigmond 2004). The FH1 domain binds profilin and recruits actin monomers to incorporate them into the growing tip of the actin filament (Romero, Le Clainche et al. 2004). In addition to the prototypical functions ascribed to RhoA, RhoA has also been shown to be active at the leading edge of lamellipodia, where it is thought to activate formins such as mDia (Machacek, Hodgson et al. 2009). Formins also directly bind microtubules, and this interaction regulates microtubule stability downstream of polarity cues ((Palazzo, Cook et al. 2001), reviewed in (Bartolini and Gundersen 2010)).

1.4.2.2 Cdc42

Initial studies analyzing the effects of dominant active and negative mutants of the Rho GTPases on cell migration demonstrated that Cdc42 is the master regulator of cell polarity. Cdc42 is active towards the front of the cell and both inhibition and global activation of Cdc42 disrupt the directionality of migration. During migration, Cdc42 is responsible for orienting the MTOC as well as the Golgi apparatus in front of the nucleus toward the leading edge (Allen, Zicha et al. 1998, Nobes and Hall 1999). At the cell front, Cdc42 functions by recruiting and activating a cytoplasmic Par6/atypical protein kinase C (aPKC) complex, with localized aPKC acting through the microtubule motor protein dynein, leading to MTOC orientation through local inhibition of GSK3β and accumulation of adenomatous polyposis coli protein (APC) at the plus
ends of microtubules resulting in centrosome reorientation (Etienne-Manneville and Hall 2001, Etienne-Manneville and Hall 2003). Here the MTOC and Golgi have been described to be maintained at the cell centre through the microtubule-dynein interaction, while Cdc42 induces actomyosin-dependent rearward nuclear movement, together producing MTOC orientation (Gomes, Jani et al. 2005). Cdc42 and Rac are also the predominant direct upstream signaling molecules of the p21-activated kinases (PAKs), a family of serine/threonine protein kinases. PAKs activated by Rac/Cdc42 mediate cytoskeletal organization and dynamics to induce formation of lamellipodia, filopodia, membrane ruffles, stress fibres and remodeling of focal adhesion complexes (reviewed in (Bokoch 2003)). Cdc42 also functions in actomyosin contractility through phosphorylation and inactivation of myosin light chain phosphatase, triggered by MRCK (myotonic dystrophy kinase-related Cdc42-binding kinase) downstream of Cdc42 mediating myosin-dependent cell motility. Further highlighting the crosstalk amongst the RhoGTPases, Rho/ROCK and Cdc42/MRCK cooperate in order to generate the actomyosin contractility needed for elongated movement through the inhibitory phosphorylation of myosin phosphatase (Wilkinson, Paterson et al. 2005). In addition, Cdc42, together with binding to phosphatidylinositol-4,5-bisphosphate (PI(4,5)P_2), binds and activates WASP and N-WASP relieving the autoinhibited conformation of WASP proteins and inducing actin polymerization through the Arp2/3 complex (Kim, Kakalis et al. 2000). WASP and N-WASP exist in an autoinhibited, closed conformation where the C-terminal WCA motif binds to the GTPase binding domain (GBD) in the inactive state.

Binding of active Cdc42 and Rac1 to the CRIB motif within the GTPase binding domain, and subsequent cooperative activation by interaction with PI(4,5)P_2 releases the WCA from the GBD, allowing for binding of the Arp2/3 complex to the WCA motif and subsequent activation.
of the Arp2/3 complex. Therefore, maximally activated N-WASP requires not only Cdc42 but also acidic phospholipids that bind to the basic domain of its amino terminal region for its function, including localization of N-WASP beneath the cytoplasmic membrane (Mullins 2000). Cdc42 is also the main GTPase regulating filopodia formation, acting through mDia formins and WASP-mediated activation of Arp2/3 (Peng, Wallar et al. 2003). Finally, Cdc42 and Rac1 bind IQGAP1 promoting microtubule capture (targeting the microtubule plus ends to certain regions of the cell cortex or to kinetochores), which is important for polarized cellular processes such as directional migration and alignment of mitotic spindles (Fukata, Watanabe et al. 2002).

1.4.2.3 Rac1

Rac promotes actin polymerization and integrin adhesion complex assembly at the cell leading edge, stimulating formation of lamellipodia and membrane ruffles, and is essential for the migration of cells (Small, Stradal et al. 2002). While Rac1 and Cdc42 have unique functions, they have also been shown to have many overlapping functions in actin remodeling. The mechanisms through which Rac catalyzes actin polymerization involve PAK protein kinases/LIMK (key roles in integrin based adhesion turnover), phosphoinositol phosphates (PIPs), the adaptor molecule IRSp53 and the Arp2/3 complex. Rac1 induces activation of the Arp2/3 complex through binding to the SCAR/Wave Regulatory Complex (WRC) components Sra1 and WAVE1 which generates a conformational change that unmasks the WCA motif in WAVE1 leading to the activation of the Arp2/3 complex and actin assembly (Eden, Rohatgi et al. 2002, Chen, Borek et al. 2010). Unlike WASP, WAVE proteins do not have a GBD domain and their activation requires binding of Rac1 to the adapter molecule IRSp53 (insulin receptor tyrosine kinase substrate p53), followed by binding of this complex to Scar/WAVE (Miki, Yamaguchi et al. 2000). Concomitantly with WAVE-mediated Arp2/3 activation, Rac-dependent signaling
recruits and activates Arpin to the lamellipodia where it binds to and acts as a competitive inhibitor of the Arp2/3 complex; this leads to a reduction in migration speed and a subsequent change in direction, controlling directionality of migrating cells (Dang, Gorelik et al. 2013). Rac also antagonizes Rho-mediated signaling through a WAVE-dependent mechanism, and through production of reactive oxygen species, which promotes activation of p190RhoGAP (Nimnual, Taylor et al. 2003).

1.4.3 Rho GTPases in cancer

Due to multiple activities of the Rho GTPases in functions including cell cycle progression, regulation of gene transcription, proliferation, cell polarity, cell adhesion and plasticity of cell migration, it is not surprising that they play a role in cancer initiation and progression. In particular, the ability of Rho GTPases to modulate epithelial cell-cell contacts, MMP expression and the mode of cell migration indicates a central role in cancer cell invasion and metastasis (Sahai and Marshall 2002). In general, no mutations in Rho proteins have been described, save for RhoH, a Rho family member, which has been reported to be genetically altered in non-Hodgkin’s lymphomas and multiple myeloma (Preudhomme, Roumier et al. 2000). Instead, deregulation of Rho GTPase signalling occurs largely at the level of expression or activation of Rho GTPases, and through expression level or activation of their regulators or downstream effectors.

Increased expression or activity of RhoA is frequently described in human tumours, including hepatocellular carcinoma (HCC) (Li, Ji et al. 2006), head and neck squamous cell carcinomas (Abraham, Kuriakose et al. 2001), breast and colorectal cancers (Fritz, Just et al. 1999). In HCC, increased RhoA expression and activity correlated with recurrence and worse prognosis (Li, Ji et
al. 2006). Increased levels of RhoA have also been shown to correlate with progression of ovarian (Horiuchi, Imai et al. 2003), bladder (Kamai, Tsujii et al. 2003), gastric (Pan, Bi et al. 2004), esophageal squamous cell (Faried, Faried et al. 2007) and testicular cancers (Kamai, Yamanishi et al. 2004). Similar to RhoA, RhoC is upregulated in many cancers and has also been proposed to be a marker for poor prognosis in several cancer types, including breast (Fritz, Just et al. 1999, van Golen, Davies et al. 1999) pancreatic ductal adenocarcinoma (Suwa, Ohshio et al. 1998), hepatocellular (Wang, Yang et al. 2004), ovarian (Horiuchi, Imai et al. 2003), bladder (Kamai, Tsujii et al. 2003), gastric (Kondo, Sentani et al. 2004), esophageal squamous cell (Faried, Faried et al. 2007), head and neck squamous cell (Kleer, Teknos et al. 2006), prostate (Iiizumi, Bandyopadhyay et al. 2008) and non-small cell lung (Shikada, Yoshino et al. 2003) carcinomas. In contrast to RhoA and RhoC, RhoB expression is often downregulated in human tumours (reviewed in (Gomez del Pulgar, Benitah et al. 2005)), and it has been proposed that RhoB may act as a tumour suppressor. Indeed, RhoB knock-out mice develop normally but have enhanced carcinogen-induced skin tumour formation (Liu, Rane et al. 2001).

Rac1 is similarly overexpressed in a variety of human tumours, including oral squamous cell carcinomas (Liu, Yen et al. 2004), and overexpression correlates with progression of testicular (Kamai, Yamanishi et al. 2004), gastric (Pan, Bi et al. 2004) and breast cancers (Schnelzer, Prechtel et al. 2000). While Rac1 knock-out in mice is embryonic lethal, conditional deletion of Rac1 in a lung cancer model showed that Rac1 function is required for K-Ras driven proliferation and tumourigenesis (Kissil, Walmsley et al. 2007). Similarly, mice deficient in the Rac-specific GEF, Tiam1, are resistant to Ras-induced skin tumours (Malliri, van der Kammen et al. 2002). Rac3, another member of the Rac subfamily, is also overexpressed in some tumours, with increased active Rac3 in breast cancers (Mira, Benard et al. 2000). Mice overexpressing
Rac3 do not display a tumourigenic phenotype, and Rac3 null mice develop normally with improved survival in mice with Brc/Abl-induced lymphomas (Cho, Zhang et al. 2005). Rac2 is expressed in the hematopoietic system, where it is required for neutrophil migration but is dispensable for migration in macrophages (Roberts, Kim et al. 1999, Wheeler, Wells et al. 2006). Rac1 is similarly dispensable for migration in macrophages (Wheeler, Wells et al. 2006), although it is required for invasion; in breast cancer and glioma cells Rac1 suppression by RNAi strongly inhibits lamellipodia formation, migration and invasion (Chan, Coniglio et al. 2005). Rac1b, the splice variant of Rac1, which does not bind RhoGDI and is therefore present predominantly in a GTP-bound state and is less susceptible to ubiquitination and degradation, is also upregulated in colorectal (Jordan, Brazao et al. 1999) and breast cancers (Schnelzer, Prechtel et al. 2000).

Cdc42 is overexpressed in breast and testicular cancers, and has been correlated with testicular carcinoma progression (Fritz, Just et al. 1999, Kamai, Yamanishi et al. 2004). While a tumour promoting role for Cdc42 has not been shown in mouse models, it has been suggested that Cdc42 contributes to tumour progression by activating Rac.

1.5 Modes of cell motility and plasticity of tumour cell migration – Epithelial to mesenchymal transition

Cell migration is governed by a complex network of signal transduction pathways that involve lipid second messengers, small GTPases, kinases, cytoskeleton-modifying proteins, and motor proteins. Different modes of cellular motility have been described, including mesenchymal, amoeboid or collective migration. In cancer, mesenchymal motility has been well-studied, and it is estimated that up to 40% of carcinomas utilize this form of motility and undergo an epithelial...
to mesenchymal transition (EMT). The epithelial-mesenchymal transition, and its reverse process-mesenchymal-epithelial transition (MET), was first recognized as a feature of embryogenesis, including in gastrulation and neural crest formation. EMT is encountered in three different biological settings, and has thus been classified as developmental (Type I), associated with tissue regeneration and organ fibrosis (Type II) and associated with cancer progression and metastasis (Type III) (reviewed in (Kalluri and Weinberg 2009)). The metastatic process requires several steps, including acquisition of increased motility by proliferating tumour cells and invasion, and the molecular mechanisms involved have been shown to involve deregulation of the signaling pathways that control EMT. Activation of the EMT program in some systems is necessary for the dissemination and invasion of cancer cells. During this process, the epithelial cells lose their apico-basal polarity and cell–cell adhesion and acquire mesenchymal properties such as migratory and invasive characteristics by multiple pathways, not all of which are known.

Shift in expression profiles of certain proteins and characteristic cytoskeletal features are often considered as biomarkers of EMT. Mesenchymal morphology is characterized by an elongated cell morphology with established migratory front-rear cell polarity, and is dependent upon proteolysis to degrade the extracellular matrix. In contrast, epithelial cells establish close contacts with their neighbors and an apicobasal axis of polarity through adherens junctions, gap junctions, and tight junctions. They express high levels of the cell surface protein E-cadherin, while mesenchymal cells express N-cadherin, fibronectin and vimentin. Loss of E-cadherin, a cell-cell adhesion molecule and negative regulator of the canonical WNT signaling cascade, in particular of its central mediator β-catenin, is considered the fundamental step in EMT. Cell lines with loss of E-cadherin show increased invasiveness and metastasis, and while E-cadherin
expression varies in different tumours, decreased expression generally correlates with worse survival (reviewed in (Birchmeier and Behrens 1994, Hirohashi 1998)). Epigenetic inactivation and mutations in the E-cadherin gene, CDH1, have been identified in cancer cells, promoting EMT and metastasis (reviewed in (Schmalhofer, Brabletz et al. 2009). Approximately 40% of individuals with diffuse hereditary gastric cancer have driver germline mutations in CDH1. This predisposes carriers to lobular breast cancer and diffuse gastric cancer, a highly invasive tumour characterized by early onset, late presentation and a poor prognosis, with strong penetrance typified by a cumulative incidence of gastric cancer by 80 years approximately 60-70% (Hansford, Kaurah et al. 2015).

In addition, acquisition of a mesenchymal phenotype is driven by several key EMT-inducing transcription factors, such as Snail1 and Snail2/Slug, Twist, Goosecoid, E12/47 and members of the zinc finger homeobox family of repressors (ZEB1 and ZEB2), which bind the E-cadherin promoter to repress its transcription or repress E-cadherin indirectly (reviewed in (Yang and Weinberg 2008)). These EMT-inducing transcription factors have been shown to be enriched in tumours, and their expression has been associated with EMT, decreased E-cadherin expression and tumour invasion and metastasis (expression in human cancers reviewed in (Peinado, Olmeda et al. 2007)). In keeping with this, it has been suggested that these transcription factors have prognostic value for cancer metastasis (Tania, Khan et al. 2014). The EMT program is induced by several signaling pathways, including not only TGF-β, receptor tyrosine kinase pathways (e.g. EGF, FGF), Notch and Wnt/β-catenin, which have been shown in cancer cells to activate the EMT-inducing transcription factors such as Snail1, Snail2/Slug, and ZEB1, thereby repressing E-cadherin expression. Loss of E-cadherin itself promotes Wnt signaling. Activation of the Wnt pathway causes upregulation of Snail and vimentin, and correlates with poor prognosis and
advanced stages of breast cancer (Blanco, Moreno-Bueno et al. 2002). While TGF-β acts as a suppressor of epithelial cell proliferation and thus suppresses primary tumourigenesis at early stages of cancer, it has also been shown to promote malignant properties of tumors, acting as a positive regulator of invasion and metastasis at the later stages, inducing EMT in some cancer cells (Oft, Heider et al. 1998, Yang, Pan et al. 2006). In particular TGF-β activates expression of Snail1, Slug/Snail2 and ZEB2 to regulate EMT (Comijn, Berx et al. 2001, Peinado, Quintanilla et al. 2003), and has been shown to contribute to murine breast cancer bone metastasis (Kang, He et al. 2005).

In mediating the EMT program, a variety of intracellular signaling networks have been described, including Ras-MAPK, PI3K, SMADs, RhoB, as well as integrins (reviewed in (Thiery 2002)). For example, Ras-MAPK has been shown to activate Snail1 and Snail2/Slug, where Snail2/Slug induces desmosome dissociation, cell spreading, and initiation of cell separation (Savagner, Yamada et al. 1997). Noncoding microRNAs, such as miR200 and miR205, have also been shown to be involved in regulating the EMT program by inhibiting the repressors of E-cadherin expression, ZEB1 and ZEB2 (Gregory, Bert et al. 2008, Korpah, Lee et al. 2008, Park, Gaur et al. 2008). In conjunction, p53 represses EMT by activating expression of various microRNAs, including miR200 and miR34, which inhibit ZEB and Snail1 expression, to maintain an epithelial phenotype (Chang, Chao et al. 2011). A link between EMT and the induction of stem-cell-like properties has also been shown, where it has been reported that Snail2/Slug is a master regulator of mammary stem cells and cancer stem cells (Guo, Keckesova et al. 2012). Nevertheless, the relationship between EMT and invasion/metastasis may be highly variable based on the cellular milieu. Two recent studies in fact showed that in breast and pancreatic cancer, EMT is dispensable for metastasis but contributes to chemoresistance.
(Fischer, Durrans et al. 2015, Zheng, Carstens et al. 2015), further highlighting the complexity inherent in this cellular process.
Aims/Hypotheses

Hypothesis:

I hypothesize that Plk4 facilitates tumour progression by enhancing cancer cell motility.

Specific Aims:

1. Describe the effect of Plk4 on fibroblast and cancer cell migration and invasion
2. Determine the effect of Plk4 on cancer cell invasion and metastasis in vivo
3. Determine the mechanism of enhanced motility by Plk4
   a. Use a proteomic approach to identify novel Plk4 interacting proteins that regulate cell motility
   b. Validate Plk4 interaction with candidate protein(s) using reciprocal co-immunoprecipitation, and confirm the specificity of the interaction by mapping the minimum domain for interaction using Plk4 deletion constructs
   c. Explore functional interaction of candidate protein(s) with Plk4
Chapter 2: Methods

2.1 Cell culture, transfection

Cells were grown at 37°C in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HeLa, MDA-MB-231, MEFs, HEK293T), Roswell Park Memorial Institute medium-1640 (RPMI) +10% FBS (MDA-MB-435) and McCoy’s 5A medium +10% FBS (U2OS).

All siRNA transfection was done using Lipofectamine RNAiMAX (Invitrogen). Cells were seeded onto 6-well plates containing 300µl of OptiMEM solution with 30nM siRNA (Dharmacon) and 4.5µl of Lipofectamine RNAiMAX. At 24h intervals, the media was aspirated and OptiMEM/siRNA/Lipofectamine RNAiMAX was added dropwise to the cells for further depletion, until 48 or 72h. A pool of four siRNAs and the individual constructs directed at Plk4 (5’-3’): GAAAUGAACAGGUAAUCUA (siRNA-A), GAAACAUCCUUCUAUCUUG (siRNA-B), GUGGAAGACUGUAUGUAUA (siRNA-C), and GGACCUUAUUCACCAGUUA (siRNA-D) (MU-005036-02), Arp2 (5’-3’): GAAGUUAACUACUGUGG (siRNA-01), GCAAGUGAUAUGUAUGUA (siRNA-02), GAAACGUGUUCGCAUGAUUA (siRNA-03), UGGUGUGACUGUUGAUAA (siRNA-04) (MU-012076-01), and the Luciferase GL2 Duplex (5’-3’): CGTACGCGGAATACTTCGA (D-001100-01) were purchased from Dharmacon and utilized.

Transient transfection was performed using PEI (Polyethylenimine) or Lipofectamine 2000 (Invitrogen). For Lipofectamine, cells were seeded onto 6-well plates and transfected with 2-3µg DNA and 5µl Lipofectamine 2000 in 500µl OptiMEM (gibco).
2.2 Plasmid constructs

All the vectors constructed in these experiments were done using the Gateway system (Invitrogen, USA) according to manufacturer instructions. The pDONR223 and pDONR201 donor vectors, and destination vectors with pcDNA 5/FRT/TO C- or N-terminal 3xFlag-tags, N-terminal BirA-Flag-tag and N-terminal mCherry-tag were kindly provided by Dr. Anne-Claude Gingras’ laboratory. The Plk4 Non-Degradable (AA) mutant, which includes two mutations within the phosphodegron, Ser293A and Thr297A, was synthesized from the Plk4 entry vector (ENSG00000142731, Gateway) using PCR-based site-directed mutagenesis (QuikChange II Site-Directed Mutagenesis Kit, Agilent Technologies).

For deletion mutant cloning, 0.5µM primers and 250ng template DNA were used to obtain N-, C-terminal and mid-sequence Plk4 fragments by PCR with 1.0U Phusion High Fidelity DNA Polymerase (M0530, New England Biolabs) in a 50µl reaction mixture. Cycling parameters for PCR were: 40 cycles, consisting of 30s at 98°C for denaturing, 35s at 60°C for annealing, 40s at 72°C for extension, and 7min at 72°C for final extension. Oligonucleotide primers to generate attB-PCR products are listed in the table below. PEG precipitation using half-volume 30% PEG 800/ 30mM MgCl2 was performed to purify the attB-PCR products. BP recombination was according to the manufacturer’s instructions using the pDONR223 entry vector. All constructs were validated by sequencing. Expression vectors were generated by recombination between the entry clones with the N-terminal 3xFlag destination vector.
List of primers for Plk4 mutant cloning

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<thead>
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<th>Forward attB1 (5’-3’)</th>
<th>Reverse attB2 (5’-3’)</th>
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<tr>
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The Arp2 T237A, T238A and T237/238A mutants, were synthesized from the Arp2 entry vector (Gateway) using PCR-based site-directed mutagenesis (QuikChange II Site-Directed Mutagenesis Kit, Agilent Technologies), according to the manufacturer's protocol and cloned into the pDEST mCherry destination vector (Gateway). Constructs were validated by sequencing.

List of primers for Arp2 mutant cloning

<table>
<thead>
<tr>
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<th>Reverse (5’-3’)</th>
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<td></td>
<td>CTTATACACTCCC</td>
<td>CCAGTTTCTGCTC</td>
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</table>
2.3 Generation of stable cell lines

Stable cell lines were generated as Flp-In HEK293 T-REx or Flp-In HeLa T-REx cell pools or clones. Flp-In HEK293 and HeLa T-REx host cell lines were maintained in DMEM+10% FBS and 100µg/mL Zeocin. The jetPRIME DNA transfection reagent (Polyplus) was used to transfect cells grown in 6 well plates with 300ng DNA (3×Flag, BirA*-Flag, Flag-Plk4, BirA*-Flag-Plk4 or BirA*-Flag-Plk4AA) and 3µg pOG44 overnight, as per the manufacturer’s instructions. Transfected cells were expanded to 6 mm plates and selected using 200µg/mL hygromycin (Multicell, 450141XL).

HeLa, MDA-MB-231, MDA-MB-435 or U2OS cell lines with stable knockdown of Plk4, FAM46C or control (Luciferase or RFP) were generated through lentiviral infection, using two-four individual short hairpin RNA (shRNA) constructs (Plk4: SHCLNG-NM_014264, Sigma; FAM46C: SHCLNG-NM_017709, Sigma). Lentiviruses were produced as described (Moffat, Grueneberg et al. 2006), and were used to infect the cells for 24h, followed by puromycin- (1 mg/ml for HeLa, MDA-MB-231 or U2OS cells, and 5 mg/ml for MDA-MB-435 cells) mediated drug selection for 7 days.

2.4 RNA Extraction and Real-Time RT-PCR

RNA was isolated using the RNEasy mini kit (QIAGEN), treated with RNase-free DNase (Invitrogen) and reverse transcribed with SuperScriptII reverse transcriptase (18064-014, Invitrogen) using Random Primers (48190-011, Invitrogen) according to manufacturers’ instructions. Real time RT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7900HT apparatus, and normalized to control endogenous HPRT, GAPDH or RPII as indicated.
Plk4<sup>+</sup> and Plk4<sup>−</sup> MEFs were generated and cultured as described (Hudson, Kozarova et al. 2001, Ko, Rosario et al. 2005, Rosario, Ko et al. 2010, Rosario, Kazazian et al. 2014).

Validation of array results for MMP-3, MMP-13, HOXA7, SCN1B and CDC42BPB in MEFs was by real-time RT–PCR, using the below primers. Three replicates were run per embryo for each run. Cell pellets from HeLa, MDA MB-231, MDA MB-435, U2OS cell lines were also collected for real time RT-PCR using the below primers. Data generated by PCR software (SDS 2.2.2, Applied Biosystems, Foster City, CA, USA) were analyzed using the 2<sup>−ΔΔCt</sup> method (Livak and Schmittgen 2001).

RT-PCR Primers (5'-3')

<table>
<thead>
<tr>
<th></th>
<th>Mus musculus</th>
<th>Homo sapiens</th>
</tr>
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<tbody>
<tr>
<td>CDC42BPB</td>
<td>F: CAGCGATGAGAAAGATGCGAGAG; R: TCCGACGAACTTCCAGAGTGG</td>
<td>Arp2</td>
</tr>
<tr>
<td>HOXA7</td>
<td>F: ACAGCGCTTTTTGAAAATATTG; R: GGGTGCAAAGGGAGCAAGAG</td>
<td>E-cadherin</td>
</tr>
<tr>
<td>HPRT</td>
<td>F: AAAACATGAGAACTTTTGAGTCCTTTCC; R: CGTCTCTTTCACCAGCAAGCT</td>
<td>FAM46C</td>
</tr>
<tr>
<td>MMP3</td>
<td>F: ACATGGAGACTTTGTCCCTT; R: GGCTGAGTGTGAGTCCC</td>
<td>Fibronectin</td>
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<tr>
<td>MMP13</td>
<td>F: CTTCTTCTTGGAGTCTGGAAGTC; R: CTTGGAAGGTCACTGTAAGACT</td>
<td>GAPDH</td>
</tr>
<tr>
<td>SCN1B</td>
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<td>MMP2</td>
</tr>
<tr>
<td>MMP3</td>
<td>F: AATCCATGAGGCAAGGCGATT; R: CATTGGTGCTAGAATCCAGA</td>
<td>MMP3</td>
</tr>
<tr>
<td>MMP13</td>
<td>F: TCAGGATGCCATGCTGACA; R: AGGGCAATCAATGGGATGAG</td>
<td>MMP13</td>
</tr>
<tr>
<td>N-cadherin</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<td>Vimentin</td>
</tr>
<tr>
<td>Vimentin</td>
<td>F: GAGAATTTTCCGCGGAGGAG</td>
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</tbody>
</table>

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2.5 Immunofluorescence

For immunofluorescence, cells were fixed using 4% paraformaldehyde (PFA) for 10min, permeabilized with 0.5% Nonidet-P40 (NP-40; Bioshop) for 20min, and blocked in 10% FBS/PBS for 1h. Antibody incubations were then conducted in the blocking solution for 1h. Staining was performed with the following antibodies FLAG M2 (mouse, 1:5000; Sigma), centrin (mouse, 1:250, clone 20H5; Millipore), pericentrin (rabbit, 1:400; Sigma), Arp2 (rabbit, 1:200, H-84; Santa Cruz Biotechnology Inc.). Secondary antibodies were conjugated to Alexa Fluor 488, 546 or 633 (1:400, Life Technologies). Samples were counter-stained with Alexa-546-phalloidin (1:400), Alexa-488-phalloidin (1:400) or Alexa-633-phalloidin (1:400). YFP and mCherry were visualized directly. DNA was detected using Hoechst, and cells were mounted in Immuno-mount medium (Thermo). Immunofluorescence images were collected using the Olympia Deconvolution fluorescence microscope and softWoRx software (Applied Precision). Images were collected using a 100× or 60× 1.4 NA oil objective (Olympus). Centriole numbers were determined by counting the number of structures that stained positive for YFP-Plk4 or centrin.

2.6 Isolation of cell protrusions and quantification using BCA assay

P3 Plk4+/+ MEFs (series 26.9, 27.1), Plk4−/− MEFs (series 26.11, 27.2), and HeLa cells were grown at 37°C in DMEM+10% FBS. HeLa cells were transfected with siRNA X48h (siGENOME set of three individual Plk4 siRNA constructs that did not alter cell viability or proliferation, or Luciferase siRNA (Dharmacon)), or transiently transfected with Flag-Plk4 or Flag control X16h. Pseudopod isolation was performed as previously described (Nguyen et al., 2000). Cells were serum-starved X8h, then 500,000 cells were plated on 1µm pore size filters
(PET membrane 6-well cell culture insert, Falcon) in serum-free media, with DMEM+20%FBS in a bottom chamber underneath the filter. After 6h, filters were washed with cold PBS and the bottom (pseudopod fraction) and top (cell body fraction) of the filter scraped with razor blades. The pseudopodia or cell body fractions were recovered from the razor blades using 70µl of cold TNTE lysis buffer (20mM Tris-HCl, pH 7.5, 120mM NaCl, 1mM EDTA, and 1% Triton-X 100) supplemented with protease inhibitors and 5mM NaF phosphatase inhibitor. Extracts were centrifuged at 14,000 rpm for 30 min (4°C), and the supernatant collected and protein concentration measured using the BCA protein assay (Pierce). For SDS-PAGE and immunoblotting, 10-15µg of protein was separated by SDS-PAGE and blocked with 5% milk-PBST, prior to probing with primary antibodies (Hsp-70 (1:5000, Affinity bioreagents) and β-tubulin (1:5000, Sigma)) and secondary anti-mouse antibody (1:10,000, GE Healthcare) conjugated to horseradish peroxidise. Labeled bands were revealed by chemiluminescence and exposed to CL-X Posure film (Thermo Scientific). For immunofluorescence, filters were fixed and stained with Alexa-546-phalloidin and Hoechst. Filters were mounted under cover slips with Immuno-mount medium (Thermo). Cells were visualized using indirect immunofluorescence performed with the Olympia Deconvolution fluorescence microscope using 40X objective, and z-stacks collated using SoftWoRx software (DeltaVision).

2.7 Rescue experiment for cell spreading with Flag-Plk4 and kinase dead-Flag-Plk4 K41M

Late passage Plk4−/− MEFs were grown at 37°C in DMEM+10% FBS. 70,000 P15 Plk4−/− MEFs (series 1.1 and 3.2) were seeded onto 6-well plates and transfected with 3µg DNA for each of the tagged constructs (Flag, Flag-Plk4 or Flag-Plk4 K41M). Cells were then trypsinized
and 35,000 cells for each condition were plated onto cover slips and allowed to spread for 90 min. Subsequently, the cover slips were washed with PBS and cells fixed and stained. Cells were visualized using indirect immunofluorescence performed with the Olympia Deconvolution fluorescence microscope using 20X and 40X objectives. Images were quantitatively analyzed using the Columbus Image Analysis System (Perkin Elmer): Hoechst-stained nuclei were detected, followed by segmentation of cell borders based on F-actin signal, and determination of cell area.

2.8 Active Plk4 pS305 localization to protrusions and centrioles in spreading assay

P3 Plk4+/+ MEFs were grown at 37°C in DMEM+10% FBS. 135,000 cells were seeded onto 6-well plates and transfected with SMARTpool murine Plk4 siRNA or Luciferase siRNA (Dharmacon) X72h. Cells were then trypsinized and 35,000 cells for each condition were plated onto cover slips and allowed to spread for 2h. Cells were then fixed and primary antibody incubation was carried out overnight at 4°C using anti-Plk4 pS305 (1:150), followed by counter staining with Hoechst, Alexa-546-phalloidin and secondary anti-rabbit Alexa Fluor 488 (1:400; Molecular Probes) for 1h at room temperature. Cells were visualized using indirect immunofluorescence performed with the Olympia Deconvolution fluorescence microscope using the 100X objective.

2.9 Wound healing migration

Wound-healing migration was assayed using confluent cells in 6-well plates and monitored using time-lapse microscopy. The following cell lines were utilized: P3 Plk4+/+ (s27.1, 26.9), P3 Plk4+/− (s27.2, 26.11), P15 Plk4+/− (s1.1, 3.2) MEFs; HeLa 48h Luciferase or Plk4 siRNA (siGENOME SMARTpool or individual constructs -A, -B, -C) transfected cells; MDA-MB-231,
HeLa and MDA-MB-435 Plk4 or control shRNA stable cell lines; HeLa T-Rex Flag-Plk4 and U2OS T-Rex YFP-Plk4 cell lines treated with tetracycline X24h. Cells were serum starved for 18h prior to scratch. Scratches were made using a P10 pipette tip, and migration was assayed up to 24h in 1% FBS-containing media. For time-lapse experiments, cells were viewed using an inverted microscope (DMIRE2; Leica Microsystems) equipped with a motorized stage and a live cell apparatus (37°C heated and humidified chamber with 5% CO₂; Applied Scientific Instrumentation) with a 6-well plate adaptor. Data acquisition was performed over the indicated time courses. Images were collected with a 10x objective lens and captured with an ORCA Hamamatsu CCD camera. All hardware and image capture conditions were made possible, and images analyzed, using Volocity 3D Image Analysis Software (Improvision). Quantification was performed measuring total wound area in 3 fields/condition at the indicated timepoints with the same software. Measurements at each location were averaged to yield a mean wound area. The residual wound area was expressed as a percent of original, which was itself highly reproducible, subtracted from 100 to yield the % healed at each time point.

2.10 Golgi positioning during directional migration

Golgi repositioning during cell migration was determined with a wound healing assay using confluent P3 Plk4+/+, Plk4+/− MEFs and HeLa cells. 750,000 P3 Plk4+/+ and Plk4+/− MEFs (26.9, 27.1, 28.7), or 625,000 Luciferase and Plk4 siRNA transfected HeLa cells were seeded onto 6-well plates with cover slips, and scratches made using a P10 pipette tip. After 90 min for MEFs or 3h for HeLas, cells were fixed and stained using primary antibody GM130 (mouse, 1:400, BD Biosciences) for 1h at room temperature, followed by counter staining with Hoechst, 546-phalloidin and secondary anti-mouse Alexa Fluor 488 for 1h. Cells were visualized using indirect immunofluorescence performed with the Olympia Deconvolution fluorescence microscope using
the 40X objective. The analysis of the Golgi position in regard to the nucleus and direction of migration was performed in a double-blind manner using the phalloidin channel to assess cell direction.

2.11 Real Time Cell Analyzer (RTCA) Transwell invasion assay

For the RTCA assay, 2X10^5 cells (HeLa siRNA transfected cells, HeLa T-REx Flag-Plk4, MDA-MB-231 shRNA and HeLa shRNA stable cell lines) were seeded into 6-well plates and serum-starved X24h. Using the RTCA system plate (Roche), 160µl DMEM+20%FCS was added to the lower chamber as a chemoattractant. 100µl serum-free media containing 50,000 cells was added to the upper chamber in transwells that had been coated with Matrigel (0.25mg/ml). The cassette was placed in the RTCA platform and real-time impedance data were analyzed using RTCA software.

2.12 Phenotype analysis

200,000 cells treated as indicated were seeded onto 6-well plates with cover slips. At varying time points, cells on the cover slips were fixed and stained as previously described. Visualization of cells using indirect immunofluorescence was performed with the Olympia Deconvolution fluorescence microscope using 20X and 40X objectives. The INCell Analyzer 2000 was used to quantify cell shape in Plk4 siRNA transfected cells at 48h. Following 24h of siRNA transfection, cells were trypsinized and 2,000 cells were seeded onto 96-well plates containing the OptiMEM-siRNA-Lipofectamine RNAiMAX solution for a further 24h. Cells were then fixed and stained with Hoechst and Alexa-546-phalloidin, and visualization and quantification of cell axial ratio (length/width; rounded=1) was performed by the INCell Analyzer 2000.
2.13 Spreading assays

Cells were seeded into 6 well plates (150,000 cells/well) with a glass coverslip or 96-well plates (3595, Costar) (2500 cells/well) in DMEM+10% FBS. For transfection in the rescue protocol, HeLa cell lines (treated with shRNA or siRNA) in 6-well plates were transfected with 3µg plasmid DNA X16h, then seeded into 96-well plates. At the indicated times cells were fixed, and stained. 96-well plates were scanned and images were acquired on the INCell Analyzer 6000 microscope (GE Healthcare), equipped with Nikon Plan Fluor 10x/NA 0.45 objective and 2048x2048 sCMOS camera. Automated cell size measurements were performed using a custom image analysis routine for Columbus 2.3 (PerkinElmer). Hoechst-stained nuclei were detected, followed by segmentation of cell borders based on F-actin signal, and determination of cell area.

2.14 Generation of Point Mutations and siRNA Rescue

Mutagenic siRNA-resistant Flag-Plk4 constructs were generated by changing 4 nucleotides in each of the Plk4 siRNA-A, -B and –C targeting regions (A387G, C393T, T399C and A402T substitutions for siRNA-A, A303G, T306C, T309G and T312A substitutions for siRNA -B, and A939G, C942T, A945T and T948A substitutions for siRNA –C) using the following primers (5’- 3’): Plk4 siRNA-A
(F:GTATTAGAAATGTGCCATAATGGAGAGATGAATAGGTACCTAAGAATAGAGTGAAACCCTTCTCAGA;
R:TCTGAGAAGGGTTTCACTCATTCTTAAGGTACCTATTCATCTCTCCATTATGGCACCATTCTAATAC), Plk4 siRNA-B
(F:GAGTCCAAAATGAGGTGAAAATACATTGCCAATTGAATAGGTACCTAAGAATAGAGTGAAACCCTTCTCAGA;
GCTTTATAACTATT;
R:AATAGTTATAAAGCTCCAAGATTGACGGGTGCTTCAATTGGCAATGTATTTTCACC
TCATTTTGGACTC), and Plk4 siRNA-C
(F:CAAAAAAGTTAAGTATTTAGGAACGTGTGGAGATTCTATAGATAGTGGGCATGCCA
CAATTCTCCTACTG;
R:CAGTAGAAATTGTGGCATGCCACACTATCTATAGAATTCTCCACAGTTCCTAAATCT
TTACTTTTTG). Mutagenic siRNA-resistant RFP-Arp2 WT and RFP-Arp2 T237/238A
constructs were generated by changing 3 nucleotides in each of the Arp2 siRNA-02 and -03
regions using the following primers (5’-3’): Arp2 siRNA-02
(F:GGTTGGTGTAGGCAAGCGAAGTTGCGATCAACGTAGAAGTTAAC; R:
GTAACTTCTACGTGATCGCAACTCGCTTGCCTCATCACCAACC), and Arp2 siRNA-03
(F:CTCTGCTGATTTTGAAACGTGGATCTTAAAGAAAAACTGTGTGTTACG; R:
CGTAACACAGTTTTTCTTTTATCATCCGAGCTGGTTTCAAATCAGCAGAG). Mutations
were generated using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies),
according to the manufacturer's protocol. Constructs were validated by sequencing. For
transfection in the rescue protocol, 250,000 HeLa cells were seeded onto 6-well plates and
transfected with siRNA X48h (Plk4) or X36h (Arp2) as described, followed by transfection with
1µg of the respective siRNA-resistant Flag-Plk4 or RFP-Arp2 WT/RFP-Arp2 T237/238A mutant
construct X16h. Flag-Plk4 expression was confirmed using immunoblots, and RFP-Arp2
expression was confirmed with immunofluorescence.

2.15 Viability and Proliferation assays

For each cell line, 1000 cells/well were seeded into 96-well plates. For HeLa, MDA-MB-231
and MDA-MB-435 cell lines, cell proliferation and viability were assessed at 24h, and every 24h
afterward for 4 days. For HeLa cells transfected with Luciferase or Plk4 siRNAs X48h, cell
viability and proliferation were assessed at 48h, and every 6h afterward for a further 24h. Cells were incubated at room temperature with Hoechst (1:800) and Propidium Iodide (1:400, 1mg/mL stock) for 15 minutes. The plates were then imaged using the Celigo Cell Imaging Cytometer, with exposure times of 150,000µs and 100,000µs for the Blue (377/447nm) and Red (531/629nm) channels, respectively. A two-step analysis procedure was performed using a gating algorithm whereby cellular debris was first excluded from the total cell number based on the cell area. Subsequently, the dead cells were distinguished from the live cells based on the mean intensity of the Propidium Iodide signal.

2.16 FLAG affinity purification and mass spectrometric analysis

HEK293 and HeLa T-REx C- and N-terminal-Flag-Plk4 stable inducible cell lines were grown to 80% confluence for each AP-MS experiment. One µg/mL tetracycline was added to each plate for 24h, with 10 plates (150-mm) being used for each biological replicate. Plates were placed on ice. Following aspiration of the media, cells were washed with ice-cold PBS. One mL of ice-cold PBS was added to each 150-mm plate, and cells were scraped using a soft cell scraper. Cells were then transferred to a 50mL centrifuge tube, and centrifuged for 5 min at 1500g (4°C), discarding the supernatant. Cells were lysed by passive lysis with lysis buffer (50 mM Hepes-KOH pH 8, 100 mM KCl, 2 mM EDTA, 0.5% NP40, 2mM DTT, 10% glycerol, 10 mM NaF, 50 mM β-glycerophosphate, 5 nM okadaic acid, 5 nM calyculin A and protease inhibitors (Sigma; P8340)), assisted by freeze-thaw. Flag-Plk4 was immunoprecipitated using anti-FLAG M2 agarose beads (Sigma; A2220) for 3h at 4°C. Immunoprecipitated complexes were then eluted in NH_4OH elution buffer and lyophilized, followed by digestion with 1µg of trypsin (Sigma; T7575) at 37°C overnight. Samples were analyzed using MS, and MS/MS data were acquired. Results were uploaded into the Samuel Lunenfeld Research Institute database for
interaction proteomics, ProHits. Assistance with the AP-Mass Spec analyses was provided by our collaborator, Dr. Anne-Claude Gingras, and members of her laboratory.

2.17 BioID purification and mass spectrometric analysis

Assistance with the BioID mass Spec was provided by Christopher Go in the laboratory of Anne-Claude Gingras.

BioID MS stable cell line generation, tissue culture

Human [taxid:9606] cells (Flp-In T-REx 293 cells from S. Angers laboratory) were transfected using jetPRIME transfection reagent (Polyplus, CA89129-924). 250,000 cells were seeded/well in 2mL DMEM, supplemented with 5% FBS, 5% Cosmic calf serum and 100 U/mL Pen/Strep. Cells were transfected with 300ng pcDNA5-BirA*-FLAG-Plk4 or pcDNA5-BirA*-FLAG, and 3µg of pOG44 in 200µL of jetPRIME buffer mixed with 3µL of jetPRIME reagent as per the manufacturer's protocol X24h. Transfected cells were passaged to10 cm plates, and the following day selected for recombination with 200µg/mL hygromycin. The selection media was changed every 2–3 days until clear visible colonies were present; these colonies were then trypsinized and replated as pools. Cells were grown to 70% confluence (5 x 150mm plates) before induction of protein expression using 1µg/ml tetracycline, and 50µM biotin for protein labeling. Cells were harvested 24 hours later as follows: cells were washed once with 10ml PBS per 150mm plate and then harvested by scraping in 1ml of PBS. Cells from 5 x 150mm plates were pelleted at 400 x g for 5 minutes, and pellets frozen on dry ice.

BioID purification and data acquisition

Cell pellets were incubated at 4°C in 1:10 pellet(g): modified RIPA buffer(mL) [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1mM EDTA, 1 mM EGTA, 0.1% SDS, Sigma protease inhibitors P8340 1:500, and 0.5% sodium deoxycholate] supplemented with 1µl of
benzonase (250U), for 1 hour on a nutator. The lysates were sonicated (3 x 10 second bursts with 2 seconds rest in between) on ice at 65% amplitude. Lysates were centrifuged X30 min at 20,817 x g at 4°C. During this step, streptavidin-sepharose beads (GE Cat# 17-5113-01) were washed 3 times with 1mL modified RIPA buffer (minus protease inhibitors and sodium deoxycholate). Beads were pelleted at 400 x g for 1 minute after each wash. After centrifugation, supernatants were transferred to fresh 15mL conical tubes and a 30µL bed volume of washed beads added to each sample. Affinity purification was performed at 4°C on a nutator for 3 hours, and then the beads pelleted (400 x g, 1 min), the supernatant removed, and the beads transferred to a 1.5mL eppendorf in 1 mL modified RIPA buffer (minus protease inhibitors and sodium deoxycholate). The beads were then washed with an additional 1mL modified RIPA buffer (minus protease inhibitors and sodium deoxycholate), followed by two washes in TAP lysis buffer (50mM HEPES-KOH pH 8.0, 100mM KCl, 10% glycerol, 2mM EDTA, 0.1% NP-40), and 3 washes in 50mM ammonium bicarbonate pH 8 (ABC). Beads were pelleted by centrifugation (400 x g, 1 min) and the supernatant aspirated in between wash steps. After the last wash all residual 50mM ABC was pipetted off and proteins digested on bead.

For digestion, beads were re-suspended in 200µL of 50mM ammonium bicarbonate pH 8 with 1µg trypsin (Sigma, no. T6567), and incubated at 37°C overnight with mixing (on rotating disc). The next day an additional 0.5 µg of trypsin was added to each sample (in 10µL 50mM ammonium bicarbonate) and the samples incubated for an additional 2 hours at 37°C with mixing. Beads were then pelleted (400 x g, 1 min) and the supernatant transferred to a fresh 1.5 mL microfuge tube. The beads were then rinsed 2 times with 75µL of MS-grade H₂O (pelleting beads at 400 x g, 1min after each rinse) and combined with the original supernatant. The pooled supernatant was then centrifuged at 16,100 x g for 5 minutes and the supernatant transferred to a
new 1.5 mL microfuge tube. These samples were then dried by centrifugal vacuum evaporation. Tryptic peptides were re-suspended in 14 μl of 5% formic acid, and 5 μl was used per analysis.

Peptides were analyzed by nano-HPLC (high-pressure liquid chromatography) coupled to mass spectrometry (MS). A spray tip was formed on a fused silica capillary column (0.75 μm internal diameter, 350 μm outer diameter) using a laser puller (Sutter Instrument Co., model P-2000, program = 4; heat = 280, FIL = 0, VEL = 18, DEL = 200). C18 reversed-phase material (Reprosil-Pur 120 C18-AQ, 3 μm) in methanol was packed [10 (±1) cm] into the column using a pressure injection cell. The column was equilibrated in 6μl of 0.1% formic acid in water and connected to a NanoLC-Ultra 2D plus HPLC system (Eksigent) coupled to an Orbitrap Elite Hybrid Ion Trap-Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with a nanoelectrospray ion source (Proxeon Biosystems). The HPLC program ran the following ratios of buffer B (0.1% formic acid in acetonitrile) to buffer A (0.1% formic acid in water): 14 min at 400 μl/min with 5% buffer B, 1 min going from 400 μl/min to 200 μl/min using a linear gradient from 5 to 2% buffer B, 90 min at 200 μl/min using a linear gradient from 2 to 35% buffer B, 5 min at 200 μl/min using a linear gradient from 35 to 80% buffer B, 5 min at 200 μl/min with 80% buffer B, 3 min at 200 μl/min using a linear gradient from 80 to 2% buffer B and 12 min at 200 μl/min with 2% buffer B. The Orbitrap Elite Hybrid Ion Trap-Orbitrap mass spectrometer was operated with Xcalibur 2.0 in data-dependent acquisition mode with the following parameters: one centroid MS (mass range, 400 to 2000) followed by MS-MS on the 10 most abundant ions. General parameters were as follows: activation type = CID, isolation width = 2 mass/charge ratio (m/z), normalized collision energy = 35, activation Q = 0.25, activation time = 10 ms. For data-dependent acquisition, the minimum signal required was 1000, the repeat count = 1, repeat duration = 30 s, exclusion size list = 500, exclusion duration = 15 s, exclusion mass
width (by mass) = low 0.6, high 1.2. To minimize carry over between samples on the autosampler, the analytical column was washed three times. Each wash consists of a “sawtooth” gradient of 35% acetonitrile with 0.1% formic acid to 80% acetonitrile with 0.1% formic acid holding each gradient for 5 min, three times per gradient. Analytical column and machine performance quality control was done after the wash runs and before a new sample by loading 30fmol BSA tryptic peptide standard (Michrom Bioresources Inc. Fremont, CA) with 60fmol α-Casein tryptic digest. The HPLC program for the quality control ran the following ratios of buffer B (0.1% formic acid in acetonitrile) to buffer A (0.1% formic acid in water): 9 min at 400 µl/min with 5% buffer B, 1 min going from 400 µl/min to 200 µl/min using a linear gradient from 5 to 2% buffer B, 30 min at 200 µl/min using a linear gradient from 2 to 35% buffer B, 5 min at 200 µl/min using a linear gradient from 35 to 80% buffer B, 5 min at 200 µl/min with 80% buffer B, 5 min at 200 µl/min using a linear gradient from 80 to 2% buffer B and 5 min at 200 µl/min with 2% buffer B. All raw mass spectrometry data and downloadable identification and SAINTexpress result tables are deposited in the MassIVE repository housed at the Center for Computational Mass Spectrometry at UCSD (http://proteomics.ucsd.edu/ProteoSAFe/datasets.jsp). The dataset has been assigned the MassIVE ID MSV000079817 and is available for FTP download at: ftp://MSV000079817@massive.ucsd.edu. The dataset was also contributed to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) with identifier PXD004327. The data is password-protected until publication (password is PLK4).

**MS data analysis**
Samples analyzed on the Orbitrap Elite Hybrid Ion Trap-Orbitrap mass spectrometer were converted to mzML using ProteoWizard (3.0.4468) (Kessner, Chambers et al. 2008) and analyzed using the iProphet pipeline (Shteynberg, Deutsch et al. 2011) implemented within ProHits (Liu, Zhang et al. 2010) as follows. The database consisted of the human and adenovirus sequences in the RefSeq protein database (version 57) supplemented with “common contaminants” from the Max Planck Institute (http://141.61.102.106:8080/share.cgi?ssid=0f2gfuB) and the Global Proteome Machine (GPM; http://www.thegpm.org/crap/index.html). The search database consisted of forward and reverse sequences (labeled “gi|9999” or “DECOY”); in total, 72,226 entries were searched. Spectra were analyzed separately using Mascot (2.3.02; Matrix Science) and Comet (2012.01 rev.3; (Eng, Jahan et al. 2013)) for trypsin specificity with up to two missed cleavages; deamidation (NQ) or oxidation (M) as variable modifications; single-, double-, and triple-charged ions allowed, mass tolerance of the parent ion to 15 ppm; and the fragment bin tolerance at 0.6 amu. The resulting Comet and Mascot search results were individually processed by PeptideProphet (Keller, Nesvizhskii et al. 2002), and peptides were assembled into proteins using parsimony rules first described in ProteinProphet (Nesvizhskii, Keller et al. 2003) into a final iProphet protein output using the Trans-Proteomic Pipeline (TPP; Linux version, v0.0 Development trunk rev 0, Build 201303061711). TPP options were as follows: general options are -p0.05 -x20 -PPM -d"DECOY", iProphet options are –ipPRIME and PeptideProphet options are –pP. All proteins with a minimal iProphet protein probability of 0.05 were parsed to the relational module of ProHits. Note that for analysis with SAINT (see below), only proteins with iProphet protein probability ≥ 0.95 and a minimum of 2 unique peptides are considered, corresponding to an estimated protein level false-discovery rate (FDR) of ~0.5%.
**Interaction scoring for BioID**

Cells expressing the FLAG tag alone or a BirA*-FLAG constructs lacking a start codon were used for endogenous biotinylation negative controls; GFP-BirA*-FLAG and BirA*-FLAG expressing stable lines were used for expression of biotin ligase for additional negative controls to model promiscuous biotinylation. Significance Analysis of INTeractome (SAINT) scoring using SAINTexpress ([Choi, Larsen et al. 2011, Teo, Liu et al. 2014]; version 3.3) was used to score true positive proximity partners. SAINT probabilities computed independently for each replicate were averaged (AvgP) and reported as the final SAINT score. Here we considered as “replicate” one purification of wild type PLK4 and one purification of a PLK4 mutant in the phosphodegron (S286A/T290A). Eight negative control experiments were compressed into two “virtual” controls using the two highest spectral counts for each protein (as defined in [Mellacheruvu, Wright et al. 2013]). For the BioID-MS experiments, proteins with AvgP ≥ 0.81 were considered true positive interactions with a calculated FDR of ≤ 2%. Downloadable files and all raw mass spectrometry files are deposited in the MassIVE repository housed at the Center for Computational Mass Spectrometry at UCSD (http://proteomics.ucsd.edu/ProteoSAFe/datasets.jsp).

**2.18 Co-immunoprecipitation**

HEK 293T cells were grown to 70% confluence in 10mm plates and transfected with 10µg DNA for each of the tagged constructs for 24h using PEI transfection reagent (Sigma). Cells were lysed using TNTE lysis buffer (20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1% Triton X-100, 1 mM EDTA), with protease inhibitor cocktail, 5mM NaF and 2mM NaOva phosphatase inhibitors. Extracts were centrifuged at 14,000rpm x 10 min (4°C), and the supernatants were immunoprecipitated with pre-washed and blocked (5% bovine serum albumin (BSA) in PBS)
anti-Flag M2 affinity gel (Sigma; A2220) for 1.5h (4°C), or incubated with rabbit polyclonal anti-mCherry antibody (Abcam) for 1h followed by immunoprecipitation with Protein G Sepharose beads (GE Healthcare) for 30min. The beads were washed 5-6 times with lysis buffer supplemented with an additional 500 mM NaCl, boiled in Laemmli sample buffer and analyzed using SDS polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblot analysis.

2.19 Immunoblotting

For immunoblot analysis, protein samples were separated by SDS-PAGE, transferred onto PVDF membranes blocked with 5% milk-phosphate buffered saline-0.1%Tween (PBS-T), and then probed with primary antibodies followed by HRP-linked secondary antibodies (GE Healthcare) and detected using SuperSignal West Femto Maximum Sensitivity Substrate (34095, Thermo Scientific). The antibodies used for immunoblotting were: Hsp-70 (mouse, 1:5000; Affinity bioreagents), β-tubulin (mouse, 1:5000; Sigma-Aldrich), FLAG M2 (mouse, F1804, 1:10,000; Sigma-Aldrich), Arp2 (rabbit, 1:750, H-84; Santa Cruz Biotechnology Inc.), Arp2 phospho T237+T238 (rabbit, 1:1000, Abcam), RFP (rabbit, 1:7,500; Abcam), mCherry (rabbit, 1:7,500; Abcam), E-cadherin (mouse, 1:1000; BD Transduction Laboratories), Fibronectin (mouse, 1:1000; BD Transduction Laboratories), Cdc42 (mouse, 1:250; Cytoskeleton Inc.). Band intensity was quantified using ImageJ. For anti-phosphoArp2 T237+T238 detection, the PVDF membrane was blocked with 5% BSA-Tris buffered saline-0.1%Tween (TBS-T). Cells were pre-treated with 5µM pervanadate for 10min prior to lysis. For lambda phosphatase treatment, cells were lysed (TNTE lysis buffer prepared without phosphatase inhibitors) and incubated with 400U Lambda Protein Phosphatase in Lambda Phosphatase Buffer (P9614, Sigma) supplemented with 2mM MnCl₂ (Sigma) for 30 min at 30°C prior to SDS-PAGE.
2.2 Cdc42–GTP pull-down

The Cdc42–GTP pull-down assay was performed with assistance from Olga Brashavitskaya using HeLa Plk4 and Luciferase shRNA cell lines plated in one 10cm dish per assay. Cells were lysed in 800µl of lysis buffer (25mM Hepes, 150mM NaCl, 1% NP40, 10% glycerol, 10 mM MgCl\textsubscript{2}, 1mM EDTA, 1mM orthovanadate, 1mM PMSF, 25mM NaF and protease inhibitor cocktail tablet) and 10µl of PAK-PBD beads were used to pull-down GTP-Cdc42. Extracts were incubated with the beads for 60 min. After washing, beads were resuspended in 15µl of Laemmli buffer and processed for immunoblotting using an anti-Cdc42 (1:250; Cytoskeleton Inc.) antibody.

2.21 Drug treatments

CK-666 (SML0006, Sigma-Aldrich) was dissolved in DMSO and used at a final concentration of 50µM unless otherwise stated. Centrinone B (5690, TOCRIS Bioscience) was dissolved in DMSO and used at a final concentration of 1000nM X16h.

2.22 Xenograft studies in mice

All protocols involving mice were evaluated and approved by the Toronto Center for Phenogenomics (TCP) Animal Care Committee in compliance with the Canadian Council on Animal Care. NOD SCID / J1303 mice from The Jackson Laboratory at 5 weeks of age were injected subcutaneously in the right flank with 1.0×10\textsuperscript{6} MDA-MB-231 RFP or Plk4 shRNA cells in Matrigel (1:1 vol). Tumour growth was monitored by palpation and tumour size was measured weekly with calipers along with mouse weight. Tumour volume was calculated assuming an ellipsoid shape. Mice were sacrificed at 4-6weeks or 7-10weeks post-injection. Timepoints for sacrifice were determined based on a pilot experiment, where I observed that RFP shRNA
xenografts had formed palpable tumours that were of a size that we could manipulate and dissect at sacrifice, with enough tumour tissue available for both histology and real time PCR, at around 5 weeks post injection. This timepoint was also chosen because 3 out of 4 RFP shRNA mice showed evidence of invasion (lymphovascular invasion or muscle invasion) on histology, although no mice had distant metastases. I also followed mice to later timepoints in the pilot experiment, and found that RFP shRNA mice reached endpoints that required sacrifice (pale, hunched posture, weight loss) or died at approximately 9 weeks post injection. These mice had evidence of gross invasion of the flank tumour into the peritoneum, and peritoneal and lung metastases. In contradistinction, Plk4 shRNA xenograft mice did not show evidence of invasion or metastases at early and late timepoints. I observed that there was a modestly reduced tumour volume in the Plk4 shRNA xenografts starting at around 5 weeks post-injection. Recognizing the probability that larger tumours would be more likely to invade or metastasize regardless of the experimental conditions (RFP or Plk4 knockdown with shRNA), I initially chose as an initial time point to sacrifice the mice at 4 weeks, when there was no statistically significant difference in tumour volume amongst the conditions. However, these tumours were difficult to manipulate, contained a significant proportion of Matrigel (vs. tumour itself), and provided minimal tumour tissue for downstream analyses. I therefore identified a threshold tumour volume (~400mm$^3$), balancing these considerations with the need to match the tumours for size, and sacrificed mice when the xenograft attained this volume. This was challenging as RFP shRNA tumours could grow very rapidly between 4-6 weeks, and some mice developed skin breakdown overlying the tumour necessitating sacrifice at smaller volumes. This approach was also utilized at the later timepoints, with a threshold tumour volume (~850mm$^3$) determined based on the average size of RFP shRNA xenografts at 7 weeks in experiment 1. A range of time to sacrifice was therefore
utilized in these experiments to match tumours for size. Individual tumours were harvested and split for fixation in 10% buffered formalin phosphate (SF100-20, Fisher Scientific) for histology and immunostaining, or snap frozen in liquid nitrogen for RNA extraction. The right lung lobes, left liver lobe and any intraabdominal metastases identified were also harvested and fixed in 10% buffered formalin phosphate for histology.

For FAM46C shRNA xenografts, NCr nude (CrTac:NCr-Foxn1nu) mice from Taconic Biosciences at 5-weeks of age were injected subcutaneously in the right flank with $5 \times 10^5$ MDA-MB-435 Luciferase, Plk4 shRNA, FAM46C shRNA, Luciferase+FAM46C shRNA, Luciferase+Plk4 shRNA or Plk4+FAM46C shRNA cells. Tumour growth was monitored by palpation, and tumour size was measured weekly with calipers along with mouse weight. Mice were sacrificed at 3-weeks post-injection as tumours had reached maximal dimensions in accordance with the TCP Animal Care Committee regulations. Individual tumours were harvested and weighed, then split for fixation in 10% buffered formalin phosphate (SF100-20, Fisher Scientific) for histology and immunostaining, or frozen in liquid nitrogen for RNA extraction. The right lung lobes and left liver lobe were harvested and fixed in 10% buffered formalin phosphate for histology.

### 2.23 Histology, immunohistochemistry and microscopy

Fixed tumours, livers and lungs were processed for paraffin embedding. Paraffin-embedded sections (4µm thick) were deparaffinized in xylene and then rehydrated in graded ethanol. For tumours, serial coronal sectioning was performed at four levels, 200µm apart. Tissues were processed and stained with hematoxylin and eosin (H&E) or the indicated antibodies according to standard protocols using the Veristain Gemini Automated Slide Stainer (Thermo Scientific) at the TCP Pathology Core. Immunohistochemistry was performed with the following antibodies:
human specific rabbit anti-vimentin antibody (RM-9120-R7, Thermo Scientific; overnight incubation), rabbit anti-Ki67 antibody (1:100, RM-9106-S1, Thermo Scientific; 1hr incubation) and rabbit anti-Pan-cytokeratin antibody (1:1000, Z0622, Dako; 1h incubation). Biotinylated goat-anti-rabbit IgG (1:200, BA-1000; Vector Laboratories) and VECTASTAIN Elite Avidin/Biotin Complex kit (1:50, PK-6100; Vector Laboratories) were used sequentially to develop immunohistochemistry. Following counterstaining with hematoxylin, sections were examined with a Leica DMR upright microscope. Histology was reviewed by a Royal College-certified pathologist (H.Wu) in a blinded manner.

2.24 Real time RT-PCR of tumour tissue

For real time RT-PCR, tissue was placed in Ambion RNAlater-ICE solution and thawed overnight at –20°C, then disrupted and homogenized in RLT buffer (RNeasy mini kit Qiagen) supplemented with β-mercaptoethanol using a rotor-stator homogenizer. The lysate was centrifuged at 4°C for 10min at 14,000 rpm and the supernatant was used for RNA purification using the RNeasy mini kit following manufacturer’s protocol.

2.25 Quantification of lung micrometastasis and Ki67

MDA-MB-231 lung metastases were detected using H&E and analyzed using a human-specific vimentin antibody (RM-9120-R7, Thermo Scientific). Images were taken at 20X magnification by light microscopy, and the number and size of the lesions determined using ImageJ software. MDA-MB-231 human-vimentin-positive colonies with an area equal to or larger than 500 µm² were considered as metastases. The metastatic burden was expressed as the number and average size of metastases present in two random sections in a defined area of 1.73mm x 975µm.

For Ki67 staining, two peripheral tumour sections were examined at 40X magnification.
Nuclear staining of Ki67 was considered positive, and the percentage of positively stained nuclei was quantified using ImageJ.

### 2.26 In vitro kinase assay-Arp2

HEK293T cells were transfected with Flag-Plk4 WT, kinase-dead Flag-Plk4 K41M, Flag-Arp2 WT or Flag-Arp2 T237/238A using PEI transfection reagent (Sigma). After 24 h, cells were lysed using TNTE lysis buffer (20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1% Triton X-100, 1 mM EDTA), with protease inhibitor cocktail, 5mM NaF and 2mM NaOva phosphatase inhibitors. Extracts were centrifuged at 14,000rpm x 10min (4°C), and the supernatants were immunoprecipitated with anti-Flag M2 affinity beads (Sigma; A2220) for 1h (4°C). For Flag-Plk4 immunoprecipitation, the beads were washed three times with lysis buffer supplemented with an additional 500 mM NaCl and twice with kinase buffer (25 mM Tris HCl, pH 7.5, 25mM MgCl2, 15mM sodium glycerolphosphate, 0.5mM NaOva, 2mM EDTA, 25 mM NaF, 1 mM DTT and 1.25 µg BSA). For Flag-Arp2, the beads were washed three times with lysis buffer supplemented with an additional 500 mM NaCl, then protein was eluted with 15µg 3XFlag-Peptide (APExBIO, A6001) with gentle mixing (4°C) x 30min. The beads with bound Flag-Plk4 were then incubated with 3µg of Arp2 protein in 30µl kinase buffer containing 100µM ATP with 10µCi [$\gamma$-33P] ATP. 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 (Invitrogen), and dried using a Bio-Rad gel dryer. Phosphorylation was visualized by autoradiography (Typhoon FLA 9500, GE Healthcare).
2.27 In vitro kinase assay-FAM46C

HEK293T cells were transfected with Flag-Plk4 WT or kinase-dead Flag-Plk4 K41M using PEI transfection reagent (Sigma) and processed as above. Flag-Plk4 was incubated with 0.25 – 2.25 µg of FAM46C or FAM46A recombinant protein (0.20µg/µl) in 30µl kinase buffer containing 100µM ATP with 10µCi [γ-33P] ATP. Kinase reactions were performed at 30°C for 30 min and terminated by adding Laemmli sample buffer. Proteins were separated by SDS–PAGE gel, stained with Colloidal Blue (Invitrogen), and dried using a Bio-Rad gel dryer. Phosphorylation was visualized by autoradiography (Typhoon FLA 9500, GE Healthcare).

2.28 Spheroid invasion assay

2500 cells in 100µl of media were added to each well of a ULA 96-well round-bottomed plate (Corning #7007) containing 100µl of culture medium. A further 100µl of culture medium containing 5% Matrigel (Corning 354234) was added to the wells to encourage spheroid formation. The plate was centrifuged at 300g (4°C) X10 min and then incubated for 4 days at 37°C and 5% CO₂. Spheroids were imaged using the INCell 6000 (bright field imaging) and spheroids of the optimal size (diameter: 400-500 µm) selected for the assay. 100 µl of medium was removed from the wells and 100µl of Matrigel added. The plate was incubated for 1h at 37°C and to facilitate Matrigel solidification. Finally, 100µl of culture medium was added on top of the solidified matrix and the plate was imaged over the next 3 days using the INCell 6000 (Image Mode 3D, Slices: 41, Steps: 50).
2.29 Statistical analysis

Statistical significance and p values were assessed by analysis of variance with Bonferroni correction and Student's t tests using Prism software (GraphPad Software, La Jolla, CA). Error bars reflect SEM.
Chapter 3: Plk4 promotes fibroblast and cancer cell migration and invasion

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


3.1 Overview

The critical role played by Plk4 in centriole duplication is well acknowledged, but Plk4 also has extra-centriolar functions such as Hand1-mediated trophoblast differentiation (Martindill, Risebro et al. 2007), facilitation of Ect2-mediated cytokinesis (Rosario, Ko et al. 2010) and in acentriolar spindle assembly (Coelho, Bury et al. 2013). Recent evidence also suggests that Plk4 may play a role in clinical cancer progression, although the mechanisms remained unclear at the outset of these studies. We identified a gene expression pattern predictive of reduced motility in Plk4+/− murine embryonic fibroblasts (MEFs), and validated this prediction with functional assays of fibroblast spreading, and cell invasion. These studies further characterize the role of Plk4 in cell spreading, protrusion formation, migration and invasion, with a focus on exploring these phenotypes in cancer cells. I show that Plk4 promotes MMP-3 and MMP-13 expression and protrusion extension across porous membranes in MEFs and cancer cells. Moreover, increased Plk4 expression enhances cell spreading in a kinase-dependent manner, and endogenous active Plk4 phosphorylated at S305 localizes to the protrusions of spreading cells. Plk4 also regulates 2D wound healing migration in MEFs and cancer cells, where the development of front to rear cell polarity is impaired in Plk4-deficient cells that have been
stimulated to migrate. In keeping with Plk4’s regulation of migration and MMP expression, Plk4 depleted cancer cells show reduced invasion through Matrigel, while Plk4 overexpressing cancer cells exhibit increased invasion in vitro. These findings illustrate a role for Plk4 in regulating cell motility and may underlie an association between increased Plk4 expression and cancer progression.
3.2 Introduction

Elderly Plk4+/− mice have a 50% incidence of spontaneous tumour formation, the majority being hepatocellular carcinomas (Ko, 2005). Analysis of human HCC specimens shows LOH at the Plk4 locus in approximately 50% of patients, with an accompanying decrease in Plk4 expression in the same patients’ tumours (Rosario, 2010), while hepatomas with retention of heterozygosity at the Plk4 locus have increased Plk4 expression in tumour compared to adjacent normal liver (Rosario, 2010). Reduced Plk4 expression has also been associated with worse survival in hepatoma patients (Pellegrino, Calvisi et al. 2010, Liu, Zhang et al. 2012). On the other hand, high expression of Plk4 has been identified as a molecular predictor of aggressive behaviour as well as resistance to therapy in breast and pancreatic cancers, and Plk4 expression is elevated in several epithelial malignancies including colorectal cancer (Macmillan, Hudson et al. 2001, Finetti, Cervera et al. 2008, Agarwal, Gonzalez-Angulo et al. 2009, Mason 2011, Mason, Lin et al. 2014, Hedley personal communication). Increased Plk4 activity causes centrosomal amplification and multipolar spindle formation, which can lead to mitotic errors and chromosomal instability. At the time that I began my doctoral work, there was little experimental evidence that Plk4 could act as an oncogene, and no studies had addressed the cellular or molecular mechanisms that might underlie such an activity, other than its role in proliferation. In some recent studies, transgenic mice with inducible expression of Plk4 in a p53 null background show increased tumour formation, including lymphomas, sarcomas and skin squamous cell carcinomas (Coelho, Bury et al. 2015, Sercin, Larsimont et al. 2016). Plk4 overexpression and subsequent centrosomal amplification has now also been shown to activate Rac1, disrupt cell-cell adhesion and increase cell invasiveness in a 3D culture system (Godinho, Picone et al. 2014). Plk4 therefore appears to function dually as both a tumour suppressor and
oncogene. My work has focused on elucidating mechanisms of this oncogenic activity.

In our initial investigations into Plk4’s oncogenic behaviour, our lab performed a microarray RNA expression analysis comparing early passage (P3) Plk4+/+ and Plk4+/− MEF lines, and immortalized late passage (P15) Plk4+/+ MEF lines. In contrast to Plk4+/− MEFs, Plk4+/+ MEFs do not undergo spontaneous immortalization, while haploid levels of Plk4 result in immortalization of MEFs in culture, with the consistent development of a near-tetraploid karyotype. 659 genes differed in expression in P15 Plk4+/+ vs. P3 MEFs, which when classified by Ingenuity Pathway Analysis revealed that among the most differentially expressed genes were those predicted to decrease cell movement in late passage Plk4+/+ MEFs when compared to early passage MEFs. While the early passage, non-immortalized Plk4+/+ and Plk4+/− MEFs did not display differences in mRNA expression in this analysis, this dataset was available for further analyses comparing early passage Plk4+/+ and Plk4+/− MEFs in terms of cell motility, independent of the immortalization process. Another dataset that was available compared gene expression in banked early passage (P3) MEFs from Plk4+/− embryos that had undergone immortalization and formed tumours when injected into NOD SCID mice, with MEFs from Plk4+/− embryos that subsequently underwent immortalization but did not cause tumours. Sixty-four differentially expressed genes distinguished the Plk4+/− lines that became tumourigenic from those that did not: the most significant differences in expression were for genes that affect cell motility. In addition, we showed in functional assays that early passage Plk4+/− MEFs had reduced cell spreading and invasion in vitro when compared to early passage Plk4+/+ MEFs, with cytoskeletal reorganization impaired in Plk4+/− MEFs that were stimulated to migrate. Taken together, these observations suggested that there were differences in cell motility as a direct result of Plk4 dosage.

Cancer cells have different properties as compared with non-transformed cells, including
altered growth characteristics (immortality in culture, decreased density-dependent inhibition of growth, decreased serum or growth factor requirements for replication, loss of cell cycle control and resistance to apoptosis), loss of cell-cell and cell-matrix interaction, loss of response to differentiation-inducing signals, altered signal transduction mechanisms, changes in cell membrane structure and function, cytologic changes such as an increased nucleus/cytoplasm ratio, and the ability to produce tumours in vivo. Near the conclusion of my studies a highly selective Plk4 inhibitor, centrinone, was introduced, and its effects highlight an additional difference between cancer cells and non-transformed cells. Specifically in relation to Plk4, proliferation of normal cells was completely inhibited by centrinone, whereas proliferation of cancer cells was only partially suppressed (Wong, Anzola et al. 2015). Thus Plk4 appeared to be essential for proliferation of normal but not cancer cells, presumably based on the need for centrioles to avoid p53-dependent apoptosis in the former but not the latter. It was therefore important to establish the Plk4 dependence of motility-related phenotypes in cancer cells. The Aims of the experiments described in this chapter were to expand our understanding of the potential role of Plk4 on cancer progression by describing the effect of Plk4 on not only fibroblast but also cancer cell protrusion formation, spreading, migration, polarity and invasion.
3.3 Results

*mRNA expression patterns in Plk4+/− vs. Plk4+/+ MEFs predict reduced motility*

In functional assays, early passage Plk4+/− MEFs displayed reduced spreading and invasion compared to their wildtype counterparts (Rosario, Kazazian et al. 2014), suggesting that even in the absence of the immortalization process Plk4 may regulate cell motility. To explore the relationship between Plk4 expression level and motility gene expression without the confounding variable of immortalization, I used the RNA expression array dataset from the previously tested four P3 Plk4+/− MEF lines and twelve Plk4+/− MEF lines to compare expression of all genes predicted to regulate cell motility, as determined by the 2000-2007 Ingenuity Pathway Analysis software. Under the category designated “Cellular Movement”, a total of 22 genes were listed. Of these genes, with the false discovery rate (FDR) set at >0.01, and p set at <0.05, in the comparison of differentially expressed genes between early passage Plk4+/− vs. early passage Plk4+/+ MEFs the top three differentially expressed genes were Hoxa7 (Fold change 1.6, p=0.004), Scn1b (Fold change 1.34, p=0.03) and Cdc42bpb (Fold change 1.15, p=0.04). Using real time RT-PCR, I validated the reduced expression of Hoxa7 and Scn1b, predicted to promote motility, in Plk4+/− vs. Plk4+/+ MEFs (Figure 3.1a). Cdc42bpb mRNA expression was not significantly changed in Plk4+/− vs. Plk4+/+ MEFs (Figure 3.1a).

Expression array analysis of early passage Plk4+/− MEF lines that subsequently went on to become tumourigenic vs. the lines that were non-tumourigenic when injected subcutaneously in NOD SCID mice, similarly showed significant differences in expression of genes that affect cell motility (Rosario, Kazazian et al. 2014). After we validated these results by real time RT-PCR, measuring the expression of two of these genes, matrix metallopeptidase-13 (MMP-13) and MMP-3, in the P3-Plk4+/− MEF lines that went on to become tumourigenic compared to P3-
Plk4\(^{+/−}\) lines that did not, we evaluated the effect of Plk4 on these motility-related genes independent of the tumourigenicity process: we directly compared P3 Plk4\(^{+/−}\) to P3 Plk4\(^{+/+}\) MEFs. Interestingly, we noted reduced MMP-13 and MMP-3 expression in early passage Plk4\(^{+/−}\) compared to Plk4\(^{+/+}\) MEFs, a result that further hinted at regulation of cell motility by Plk4 (Rosario, Kazazian et al. 2014). To confirm the causal relationship of the reduced MMP-13 and -3 expression to Plk4, I transfected early passage Plk4\(^{+/−}\) MEFs with Flag-Plk4 and effected a partial rescue in MMP expression (Figure 3.1b). Similarly, in HeLa cells with Plk4 knockdown to approximately 25% of control by short hairpin RNA (shRNA), there was a \(>50\%)\) reduction in MMP-13 and -3 expression, and a corresponding increase in MMP-13 and -3 expression with Flag-Plk4 overexpression (Figure 3.1c). Taken together, the two array analyses and subsequent validation raised the possibility that Plk4 heterozygosity may result in impaired cell motility/invasion.
Figure 3.1

(a) Expression of HOXA7, SCN1B, and CDC42BPB in P3 Plk4+/+ and P3 Plk4+/- MEFs.

(b) Relative expression of MMP-3 and MMP-13 in 6.5 Flag, 6.5 Flag-Plk4, 6.6 Flag, and 6.6 Flag-Plk4 in P3 Plk4+/- MEFs.

(c) Relative expression of MMP-3 and MMP-13 in Flag and Flag-Plk4 in Luc shRNA, Plk4 shRNA-1, and Plk4 shRNA-2.
Figure 3.1. Gene expression pattern of Plk4+/- MEFs predicts reduced motility. a) 
Confirmation of decreased expression of the indicated pro-motility genes, as identified through expression array analysis comparing P3-Plk4+/− vs. P3-Plk4+/+ MEFs (n=4 embryos/genotype, *p<0.04 vs. P3-Plk4+/+). b) MMP-3 and -13 expression was shown to be reduced in P3-Plk4+/− vs. P3-Plk4+/+ MEFs. Transient transfection with Flag-Plk4 increased MMP-3 and -13 expression in MEFs from P3-Plk4+/− embryos 6.5 and 6.6 (n=2 independent experiments/embryo), and in HeLa cells (c, bottom panel, n=3 independent experiments, *p<0.03 vs. Flag), compared to Flag alone. Stable suppression of Plk4 by transduction with shRNA reduced MMP-3 and -13 expression in HeLa cells (c, top panel, n=3 independent experiments, *p<0.05, **p<0.01 vs. Luciferase).
Plk4 promotes MEF protrusion formation, migration and polarity

We performed additional functional assays, and found that early passage Plk4\(^{+/+}\) MEFs displayed reduced protrusion formation in spreading assays compared to Plk4\(^{+/+}\) MEFs (Rosario, Kazazian et al. 2014). In a different system that measured invasiveness by quantifying protrusional protein in cells invading through a porous mesh towards serum (Figure 3.2a), the abundance of protrusional protein was two-fold greater in Plk4\(^{+/+}\) compared to Plk4\(^{+-}\) MEFs, in keeping with the decreased number of protrusions observed in Plk4\(^{+-}\) MEFs (Figure 3.2b). In addition, late passage P15-Plk4\(^{+-}\) MEFs displayed markedly impaired spreading compared to early passage P3-Plk4\(^{+-}\) and Plk4\(^{+/+}\) MEFs. To confirm the specificity of the cell spreading defect to Plk4 in Plk4\(^{+-}\) MEFs, I transfected cells with Flag-Plk4. While transfection efficiency was low (approximately 5-10\%), at 2h after plating MEFs that expressed Flag-Plk4 at detectable levels had more protrusions and had spread significantly more than neighbouring cells and more than Flag-alone transfected controls, while transfection with kinase dead Flag-Plk4 K41M failed to rescue the spreading defect (Figure 3.2c). We observed Flag-Plk4 localization to the lamellipodia, a subcellular localization not previously reported. In spreading Plk4\(^{+/+}\) MEFs, a phospho S305-specific Plk4 antibody also stained the protrusions of spreading cells at 2h with depletion of Plk4 with siRNA for 24h abrogating staining in the protrusions, but not in centrioles where Plk4 protein appears more stable (Figure 3.2d).
Figure 3.2

a) P3 Plk4^{+/+} vs. P3 Plk4^{+-}

b) Protein in Protrusions

Protein: Hoechst, Phalloidin

HSP70, β-Tubulin

130kD, 70kD

P3 Plk4^{+/+}, P3 Plk4^{+-}

Protein in Protrusions

0.00
0.05
0.10
0.15

Cell Area (% of untransfected control)

Control, Flag-Plk4, Flag-Plk4 K41M

Untransfected, Luciferase siRNA

Plk4 pS305

2 h post seed

Merge

Actin

β-Tubulin

Flag-Plk4

Flag-Plk4 K41M

Merge

Control

Flag-Plk4

Flag-Plk4 K41M
Figure 3.2. Plk4 promotes fibroblast protrusion formation, spreading and localizes to the protrusions of spreading cells. a) Confocal z-sections of Plk4\(^{+/+}\) and Plk4\(^{+/−}\) MEFs 12h after plating on 1-μm pore size filters, stained with phalloidin (red) and Hoechst (blue); white line marks the position of the filter. Nuclei were not observed on the underside of the filter. b) Quantification of protrusional protein. MEFs were separated into Cell Body (CB) and Protrusion (P) fractions as in a, confirmed by immunoblot for mitochondrial Hsp70 (left panel, β-tubulin loading control, representative of n=3 experiments). Protein content in the P fraction, as a proportion of CB protein by BCA assay (right panel, n=3, \(p=0.045\)). c) P15-Plk4\(^{+/−}\) MEFs transfected with Flag-Plk4 (green) and seeded onto glass slides have a larger cell area at 90 min (*\(p=0.0036\) vs. Flag alone and vs. Flag-Plk4 K41M kinase dead mutant (green), 2 independent experiments of 2 embryos/condition, scale bar, 50μm; cell area expressed as a percent of the average area of untransfected control cells for each embryo). 30 cells scored per condition. d) Spreading Plk4\(^{+/+}\) MEFs show localization of phosphorylated Plk4 (pS305) in protrusions 2h after seeding (scale bar, 25μm), while Plk4 siRNA-treated cells lack protrusions (≈85% of cells) and show no protrusional staining (100% of cells) for pS305 Plk4. All data are mean±SEM.
Furthermore, in a wound healing assay, P15-Plk4\(^{+/+}\) MEF migration was delayed compared to early passage cells of either genotype, as was P3-Plk4\(^{+/+}\) MEF migration at 4h, 6h and 8h after wounding compared to P3-Plk4\(^{++}\) cells (Figure 3.3a, b). I also explored the development of directional polarity in migrating MEFs, where in cells moving towards a site of wounding the actin and tubulin cytoskeletons are reorganized in the direction of the wound, with the nucleus left at the trailing edge of the cell and the Golgi apparatus as a marker of the microtubule-organizing center (MTOC) localized between the nucleus and the wound. This reorientation of the Golgi was observed in over 80% of early passage Plk4\(^{++}\) MEFs at 90 min post wounding while an appropriately localized Golgi apparatus was detected in less than 50% of Plk4\(^{+/+}\) MEFs (Figure 3.3c).
Figure 3.3

(a) 0h 6h 12h 24h
P3 Plk4<sup>+/+</sup>
P3 Plk4<sup>+-</sup>
P15 Plk4<sup>+/</sup>

(b) Wound Area Healed (%)

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<th>Time (h)</th>
<th>P3 Plk4&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>P3 Plk4&lt;sup&gt;+-&lt;/sup&gt;</th>
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<td>24</td>
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(c) GM 130 Hoechst
Plk4<sup>+/+</sup>
Plk4<sup>+-</sup>

Cells with re-oriented Golgi (%)

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<tr>
<th></th>
<th>Plk4&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Plk4&lt;sup&gt;+-&lt;/sup&gt;</th>
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* p < 0.05
** p < 0.01

GM 130: a marker for Golgi localization.
Figure 3.3. Plk4 enhances migration and regulates polarity in motile MEFs. a) Scratch wound assay showing delayed healing by Plk4+/− MEFs (b shows percent of original wound area healed at the indicated time point, n=2 embryos/genotype, 4 independent experiments, *p<0.05 vs. P3-Plk4+/−, **p<0.05 vs. P3-Plk4+/− and P3-Plk4+/+. c) Location of the Golgi (white arrows) as a marker of the microtubule organizing centre (MTOC) identified by staining for GM130 (green), relative to the nucleus (Hoechst, blue), at 90min after wounding a monolayer of P3 MEFs (orientation of wound is indicated by white squares; scale bar 25µm). Right panel shows percent of P3 MEFs with appropriately localized (i.e. oriented towards the wound) Golgi (≥30 cells /experiment, n=2 embryos/genotype, 3 independent experiments,*p=0.01 vs. Plk4+/−). All data are mean±SEM.
Plk4 regulates cancer cell migration and invasion

I then went on to explore the effect of Plk4 in regulating cancer cell motility phenotypes. Depletion of Plk4 to 27-40% of control using shRNAs markedly suppressed scratch wound healing by MDA-MB-231 breast cancer cells, without affecting viability or proliferation (Figure 3.4a,b). The selective inhibitor of Plk4 kinase activity, centrinone B, similarly suppressed wound healing by MDA-MB-231 cells (Figure 3.4c). Impairment of directional migration was also observed in HeLa cells, whether acutely or stably depleted of Plk4 (Figure 3.5a,b) and in MDA-MB-435 cells with Plk4 shRNAs (Figure 3.5c). As predicted in cancer cells (Wong, Anzola et al. 2015), modest Plk4 depletion did not significantly affect viability or proliferation in these cell lines where p53 is not intact (Figure 3.6). In HeLa T-REx Flag-Plk4 and U2OS T-REx YFP-Plk4 cell lines, increased expression of Plk4 enhanced wound healing (Figure 3.7a,b and Figure 3.8a,b). Invasion across a matrigel-coated transwell membrane was impaired in Plk4-depleted MDA-MB-231 and Hela cells, both stably depleted using Plk4 shRNAs and acutely depleted using Plk4 siRNAs (Figure 3.9a-c). Three different RNAi-resistant Plk4 constructs were made against the three Plk4 siRNAs that did not alter cell viability or proliferation and tested, although one was not suitable for downstream applications as it did not rescue Flag-Plk4 protein expression subsequent to transfection with the corresponding siRNA (Figure 3.9d). The impaired transwell invasion in Plk4 siRNA transfected HeLa cells could be partially rescued by transfection with RNAi-resistant Plk4 (Figure 3.9d). Modest elevation of Plk4 levels using an inducible stable system enhanced transwell migration in HeLa cells (Figure 3.9e). In measuring protrusional protein in cells invading through a porous mesh towards serum, depletion and elevation of Plk4 levels respectively suppressed and stimulated protrusion formation by HeLa cells (Figure 3.9f).
Figure 3.4

a. MDA-MB-231

b. Relative Plk4 mRNA expression (ΔΔCt)

c. Viability and proliferation

DMSO

centrinone B
**Figure 3.4. Plk4 promotes MDA-MB-231 breast cancer cell migration.**
a) Time-lapse phase-contrast images from scratch-wound assay performed on confluent MDA-MB-231 Plk4 shRNA or RFP shRNA stable cell lines. Right panel, quantification of wound healing over time demonstrates impaired directional migration in Plk4 shRNA cell lines (data points = percent of original wound area healed at the indicated time), n=3, *p<0.0001 vs. Plk4 shRNAs. b) Left panel, relative Plk4 mRNA levels in MDA-MB-231 cells transduced with RFP or Plk4 shRNAs, n=3, *p<0.0001 vs. RFP shRNA. Right panels, viability and proliferation of MDA-MB-231 RFP or Plk4 shRNA cell lines. Cells were labeled with Hoechst and Propidium Iodide, then imaged using the Celigo Cell Imaging Cytometer at the indicated times. Dead cells were distinguished from the live cells based on the mean intensity of the Propidium Iodide signal, n=6, p=NS vs. RFP shRNA. All data are mean±SEM. c) Left panel, scratch-wound assay performed on MDA-MB-231 cells treated with centrinone B or DMSO X16h. Right panel, quantification demonstrates impaired directional migration in centrinone B-treated cells, n=3, *p<0.03 vs. DMSO.
Figure 3.5

(a) HeLa 48h siRNA

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(b) HeLa shRNA

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(c) MDA-MB-435 shRNA

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**Figure 3.5. Plk4 regulates HeLa and MDA-MB-435 cancer cell migration.** a-c) Time-lapse phase-contrast images from scratch-wound assays, showing reduced migration with Plk4 knockdown compared to control. Right panels, quantification of wound healing over time (data points = percent of original wound area healed at the indicated time). 

a) HeLa 48h Plk4 siRNA: n=5, *p<0.05, **p<0.001 vs. Luciferase siRNA. 
b) HeLa Plk4 shRNA: n=2, *p<0.001 vs. Luciferase shRNA. 
c) MDA-MB-435 Plk4 shRNA: n=3, * p<0.05, **p<0.001 vs. Luciferase shRNA. Data are means ±SEM.
Figure 3.6

HeLa 48h siRNA

Luciferase siRNA
Plk4 siRNA-A
Plk4 siRNA-B
Plk4 siRNA-C

Percent viable cells
Time (h)

HeLa shRNA

Luciferase shRNA
Plk4 shRNA#1
Plk4 shRNA#2

Percent viable cells
Time (h)

MDA-MB-435 shRNA

Luciferase shRNA
Plk4 shRNA#1
Plk4 shRNA#2

Percent viable cells
Time (h)
Figure 3.6. Plk4 knockdown in cancer cell lines does not significantly alter viability or proliferation. Viability (a, c, e) and proliferation (b, d, f) of cancer cell lines, treated as indicated. Cells were labeled with Hoechst and Propidium Iodide, then imaged using the Celigo Cell Imaging Cytometer at the indicated times. Dead cells were distinguished from the live cells based on the mean intensity of the Propidium Iodide signal. Number of independent experiments was 2 for a, b 2 for c, d 3 for e, f. Data are means±SEM. For all panels p=NS vs. control.
Figure 3.7

(a) HeLa TREx Flag-Plk4

(b) clone N1

(c) clone C1

(d) Percent cells (YFP-positive foci)
Figure 3.7. Plk4 overexpression increases HeLa migration.  

a) Relative Plk4 mRNA levels in HeLa T-REx N- and C-terminal Flag-Plk4 cells (clones N1 and C1), with Plk4 expression induced by Tetracycline. Right panel, immunoblots confirm induction of Flag-Plk4 expression; anti-Flag antibody with γ-tubulin as a loading control. 

b) Time-lapse phase-contrast images from scratch-wound assays performed on confluent HeLa T-REx Flag-Plk4 stable clones. Bottom panels, corresponding quantification of demonstrates enhanced directional migration with tetracycline induction X24h (data points = percent of original wound area healed at the indicated time). Clone N1: n=2, *p<0.05 vs. no tetracycline; Clone C1: n=3, *p<0.05, **p<0.01 vs. no tetracycline. 

c) Representative immunofluorescence images of HeLa T-REx Flag-Plk4 cells labeled with antibodies to pericentrin (red), Flag (green) and Hoechst (blue). 

d) Bar graph showing indicated number of Plk4-positive foci in cells as in c. n>80 cells, 2 independent experiments. For real time-RT PCR, RPII mRNA was used for normalization. Data are means ±SEM.
Figure 3.8

(a) U2OS TREx YFP-Plk4

(b) Relative Plk4 mRNA expression (ΔΔCt)

(c) Centrin, YFP-Plk4, Hoechst

(d) Percent cells (centrin-positive foci)

Figure 3.8

Tet (µg/mL)

Time (h)

Percent Wound Area Healed (%)
Figure 3.8. Plk4 overexpression increases U2OS cell migration. a) Relative Plk4 mRNA levels in U2OS T-Rex YFP-Plk4 cells with Plk4 expression induced by Tetracycline. b) Time-lapse phase-contrast images from scratch-wound assays performed on confluent U2OS T-Rex YFP-Plk4 cells. Right panel, quantification of wound healing over time, n=5, *p<0.0001 vs. no tetracycline. c) Representative immunofluorescence images of U2OS T-Rex YFP-Plk4 cells labeled with antibodies to centrin (red) and Hoechst (blue). d) Bar graph showing frequency of cells with indicated number of centrin-positive foci as in e. For real time-RT PCR, RPII mRNA was used for normalization. Data are means±SEM.
Figure 3.9. Plk4 promotes cancer cell invasion. a) Real Time Cell Analyzer (RTCA) transwell invasion assay results, showing reduced invasion by Plk4 shRNA vs. RFP shRNA MDA-MB-231 cell lines (representative of three independent experiments). b) Invasion by Plk4 shRNA cells is reduced vs. Luciferase shRNA as measured in RTCA transwell invasion assay (representative of two independent experiments). c) Reduced invasion in HeLa cells acutely depleted of Plk4 using siRNA vs. Luciferase siRNA X48h (representative of two independent experiments). d) Left panel, anti-Flag immunoblot confirms expression of non-degradable Flag-Plk4 mutants in HeLa cells synchronously depleted of Plk4 with the respective siRNA. β-tubulin is loading control. Right panels, RTCA transwell invasion assays show partial rescue of invasion with transfection X16h of two distinct non-degradable Flag-Plk4 mutants in HeLa cells depleted of Plk4 using corresponding siRNA constructs B and C (representative of two independent experiments). e) RTCA results showing increased invasion 24h after Flag-Plk4 expression induced in HeLa T-REx cell line with tetracycline at doses shown, vs. no-tetracycline control (representative of two independent experiments). f) Confocal z-sections of HeLa cells 12h after plating on 1µm pore-size filters (phalloidin, red; Hoechst, blue); white line marks position of the filter. Cells separated into Cell Body (CB) and Protrusion (P) fractions, confirmed by immunoblot for mitochondrial-Hsp70 with β-tubulin loading control. Quantification of protein content in P fraction, as a proportion of CB protein, shows that Plk4 siRNA reduces the amount of protrusional protein, n=3, *p<0.05, **p<0.001 vs. Luciferase siRNA, while forced expression of Plk4 increases protrusional protein, p=0.05 vs. Flag (right panels). All data are means±SEM.
Plk4 modulates cancer cell shape and spreading

Exposure of HeLa cells to increased Plk4 levels for 48h invoked a marked phenotypic change characterized by an exaggerated spindle morphology with extended protrusions reminiscent of neuronal axons (Figure 3.10a). In a spreading assay, Plk4 transfected HeLa cells formed more protrusions and attained a greater cell area than did a kinase-dead Flag-Plk4 K41M or Flag-alone transfected controls (Figure 3.10b), again implying that kinase activity is required for Plk4’s effect on cell spreading. In keeping with the defective polarity observed in Plk4+/− MEFs, transfection of HeLa cells with Plk4 siRNA without a spreading stimulus resulted in a predominantly rounded, unpolarized cell morphology (Figure 3.11a). Also, cells that were stably depleted of Plk4 with shRNA were notably deficient in spreading, a defect that was partially rescued by transient transfection with wildtype Flag-Plk4 (Figure 3.11b); Plk4 depletion by siRNA had a similar effect on spreading and could be rescued by non-degradable Plk4 mutants (Figure 3.11c, d).
Figure 3.10

(a) Flag-Plk4 WT
Flag-Plk4 K41M
Flag

(b) 3h spreading assay

Flag-Plk4 WT
Flag-Plk4 K41M
Flag

Kinase dead
Flag-Plk4 K41M
Flag

Cell Area (µm²)

<table>
<thead>
<tr>
<th>Flag</th>
<th>Flag-Plk4</th>
<th>Flag-Plk4 K41M</th>
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<tbody>
<tr>
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*
**Figure 3.10. Plk4 promotes cancer cell spreading.** a) Representative immunofluorescence images of HeLa cells transfected with Flag-Plk4 WT (wildtype, green, top panels) or kinase-dead Flag-Plk4 K41M (green, middle panels). Flag-Plk4 WT transfected cells show an arborized phenotype at 48h. Bars: 100 µm. b) Representative immunofluorescence images of spreading HeLa cells transfected with Flag-Plk4 WT (green, top panels), kinase-dead Flag-Plk4 K41M (green, middle panels) or Flag-control X18h (phalloidin, red; Hoechst, blue), showing dependence of Plk4’s stimulation of spreading on its kinase activity. Quantification of cell area (right panel). *p<0.001 vs. Flag or Flag-Plk4 K41M; n=6 experiments, 600 measured cells in each. Bar: 50 µm. Data are means±SEM. Cell area quantified using INCell Analyzer 6000.
Figure 3.11

(a) Phalloidin and Hoechst staining of cells treated with different siRNAs. Luciferase siRNA, Plk4 siRNA-pool, Plk4 siRNA-A, Plk4 siRNA-B, Plk4 siRNA-C.

(b) 3h spreading assay. Luciferase shRNA, Plk4 shRNA#1, Plk4 shRNA#2. Cell Area (μm²) plotted for each condition.

(c) Luciferase siRNA, Plk4 siRNA-pool, Plk4 siRNA-A, Plk4 siRNA-B, Plk4 siRNA-C. Cell Area (μm²) normalized to control.

(d) Plk4 siRNA-B (48h) and Plk4 siRNA-C (48h). Mean Cell Area (% normalized to control) for different conditions.
Figure 3.11. Plk4 regulates cancer cell shape and spreading.  

**a)** Representative immunofluorescence images of HeLa cells transfected with Plk4 siRNAs X48h (phalloidin, red; Hoechst, blue), showing a more rounded cell phenotype vs. Luciferase siRNA. Bar: 100 µm. Right panel, quantification of percent cells with a rounded (axial ratio > 0.8) phenotype, n=2, *p<0.01 vs. Luciferase siRNA.  

**b)** Representative immunofluorescence images of spreading Plk4 or Luciferase shRNA HeLa cell lines (phalloidin, red; Hoechst, blue). Quantification of cell area (middle panel), n=2, *p<0.006 vs. Luciferase shRNA. Quantification of cell area (right panel) in spreading Luciferase or Plk4 shRNA cell lines transfected X18h with Flag-Plk4 or Flag, showing partial rescue of reduced cell area, n=4, 500 cells measured in each, *p<0.03 vs. Flag. Bar: 100 µm.  

**c)** Representative immunofluorescence images of spreading HeLa cells transfected with Plk4 or Luciferase siRNAs X48h (phalloidin, red; Hoechst, blue). Right panel, quantification of cell area, n=4, *p<0.001 vs. Luciferase siRNA.  

**d)** Representative immunofluorescence images of spreading HeLa cells transfected with the indicated Plk4 siRNAs X48h then transfected with the respective non-degradable Flag-Plk4 mutant X16h (Flag-Plk4 mutant, green; phalloidin, red; Hoechst, blue), n=3, *p<0.05 vs. Plk4 siRNA+Flag. Bars: 50 µm. Data are means ±SEM. Cell area and axial ratio quantified using InCell-Analyzer 6000.
3.4 Discussion

The polo-like kinase, Plk4, is known as the master regulator of centriole biogenesis, with the chief subcellular domain for localization of Plk4 protein being the centriole (Bettencourt-Dias, Rodrigues-Martins et al. 2005, Habedanck, Stierhof et al. 2005). However, evidence that Plk4 has functional roles outside of centriole duplication continues to grow, such as with Hand1-mediated trophoblast differentiation in murine embryogenesis (Martindill, Risebro et al. 2007). The data presented in this chapter in conjunction with our group’s publication by Rosario et al. (Rosario, Kazazian et al. 2014) support a novel function for Plk4 in regulation of cell polarity, migration and invasion in MEFs and cancer cells.

In functional assays early passage Plk4+/− MEFs displayed reduced spreading and invasion compared to their wildtype counterparts (Rosario, Kazazian et al. 2015), suggesting that Plk4 may regulate cell motility. Here I surveyed the gene expression array dataset generated by Rosario and colleagues (2015) for differentially expressed genes predicted to regulate cell motility in early passage Plk4+/− compared to Plk4+/+ MEFs. I validated reduced expression of the three most differentially expressed genes, HOXA7, SCN1B and CDC42BPB, which are predicted to promote motility, in Plk4+/− vs. Plk4+/+ MEFs. The transcription factor, HOXA7, is overexpressed in ovarian cancer, acute myeloid leukemia and meningioma, where it is associated with worse prognosis (Afonja, Smith et al. 2000, Ota, Gilks et al. 2007, Di Vinci, Brigati et al. 2012). It stimulates proliferation in several cell types, including hepatoma and breast cancer cells, and knockdown has been shown to reduce migration (Orlovsky, Kalinkovich et al. 2011, Zhang, Cheng et al. 2013, Li, Yang et al. 2015). SCN1B, the voltage gated sodium channel β1 subunit, a member of the immunoglobulin superfamily of cell adhesion molecules, is best characterized in the central nervous system where it regulates neurite migration and adhesion
(Brackenbury, Davis et al. 2008, Brackenbury, Yuan et al. 2013). In breast cancer specifically, SCN1B has been shown to have upregulated expression, promote adhesion and migration in breast cancer cells, and increase tumour growth and metastasis formation in a xenograft model (Nelson, Millican-Slater et al. 2014). CDC42BPB, also known as Myotonic dystrophy protein kinase-like beta (MRCKß), is an important downstream effector of Cdc42 that plays a role in the regulation of cytoskeleton reorganization, modulating lamellar actomyosin retrograde flow and is crucial for cell protrusion and migration (Tan, Yong et al. 2008, Huo, Wen et al. 2011, Tan, Lai et al. 2011). While expression of these three genes was lower in Plk4 +/- vs. Plk4 +/+ MEFs, this did not achieve statistical significance for CDC42BPB. While these findings alone were not conclusive, they supported the prediction of decreased cellular movement in Plk4 deficient cells.

We then went on to functionally validate our hypothesis that Plk4 is involved in regulating cell migration by evaluating Plk4-mediated spreading, protrusion formation and migration. Plk4 +/- MEFs and Plk4 depleted cancer cells showed reduced protrusion extension through a porous mesh, reduced spreading which was kinase-dependent, reduced migration and reduced transwell invasion as compared to wildtype cells or controls. As a large number of proteins that regulate cell migration localize transiently to the leading edge of the cell at some point during the process of cell motility, we looked for Plk4 localization at the protrusions of spreading cells. Active Plk4 phosphoS305 was shown to localize to the protrusions of spreading MEFs, indicating that Plk4 may also function at this cell site to regulate cytoskeletal functions. However, how Plk4 is transported to cell protrusions remains an area for further investigation. One hypothesis is that Plk4 is shuttled via the plus end of microtubules and thereby delivered to zones of actin polymerization, similar to the centrosomal anchoring protein ninein, which is transported from centrosomes to an apical site during the development of apicobasal polarity in epithelial cells.
(Moss, Bellett et al. 2007). In this regard, Coelho et al. have demonstrated highly dynamic movement of Plk4 between spindle poles and the plasma membrane in murine embryos (Coelho, Bury et al. 2013). Other kinases that have been shown to shuttle between centrosomes and membrane protrusions include Focal Adhesion Kinase (FAK), a tyrosine kinase that functions as a critical mediator of integrin signalling. FAK localizes to focal adhesions upon cell attachment to extracellular matrix, while Ser-732-phosphorylated FAK has been found to localize to centrosomes in mitotic cells and regulate centrosome duplication and mitotic spindle formation (Park, Shen et al. 2009). In this regard it is interesting to note that several other focal adhesion proteins have been shown to localize and function both at the membrane and in centrosomes, including HEF1 (Pugacheva and Golemis 2005), paxillin (Herreros, Rodriguez-Fernandez et al. 2000), and ILK (Fielding, Dobreva et al. 2008). Nevertheless, the mechanisms by which these proteins move from the centrosome to the cell protrusions is still not clear, and can be an interesting area for future study.

Plk4’s effect on migration and invasion could be mediated by several signalling pathways, key among them the family of small Rho GTPases. Our group and others have shown that Plk4 promotes Rac1 activation (Brashavitskaya, Kazazian et al. 2013, Godinho, Picone et al. 2014), and we have similarly shown that Plk4 promotes RhoA activation (Rosario, Ko et al. 2010). Rho GTPase regulation is effected by over 80 known activating GEFs and 60 inactivating GAPs, which enable cells to reorganize their cytoskeleton in response to a variety of intra- and extracellular cues. During cytokinesis, Plk4 is shown to phosphorylate the RhoA GEF Ect2, which activates RhoA to promote cleavage furrow formation in cytokinesis (Rosario, Ko et al. 2010). While Ect2’s role in cytokinesis is recognized, a potential role in cell migration has also been suggested by identification of Ect2 in membrane ruffles of highly invasive human astrocytoma
cells, but not in benign astrocytes (Weeks, Okolowsky et al. 2012). In the protrusions of HeLa cells co-transfected with Flag-Plk4 and GFP-Ect2, Ect2 and Plk4 stain in a similar distribution (Rosario, Kazazian et al. 2015), indicating that activation of RhoA in cell protrusions by Plk4 may be effected through its interaction with Ect2. It is possible that Plk4 can interact with other GEFs or GAPs to induce activation of RhoA or Rac1 to promote cell migration, suggesting an area of further study. The failure of the Plk4+/− MEFs and Plk4 depleted HeLa cells to appropriately reorient their Golgi/MTOCs in response to a migration stimulus also implicates Cdc42 signalling defects in Plk4-deficient cells. Cdc42 is considered the master regulator of cell polarity, shown to directly orchestrate the orientation of the MTOC and the Golgi during migration (Nobes and Hall 1999). However, Cdc42 activation by Plk4 had not been explored prior to the studies described in this thesis. Signaling through pathways that stimulate actin remodelling and cell migration is also associated with gene regulation. For example, activated Rac1 can induce expression of MMP1 in fibroblasts (Kheradmand, Werner et al. 1998), and in HT1080 fibrosarcoma cells, Rac1 mediates MMP2 activation to facilitate cell invasion through collagen (Zhuge and Xu 2001). Hence, the observed reduction in MMP-3 and -13 expression associated with Plk4 heterozygosity and depletion may also be mediated by these pathways and may in turn be facilitating Plk4-mediated invasion, and requires further investigation.

In this chapter I showed that Plk4 regulates spreading, polarity, migration and invasion in cancer cell lines, in addition to nontransformed cells. This was important to establish as Plk4 appears to be differentially regulated in cancer cells compared to normal cells. For example, p53 and the SAPK pathways are frequently simultaneously inactivated in human cancer cells, and are therefore unable to regulate Plk4 activity to maintain centrosome integrity under stress (Nakamura, Saito et al. 2013). Moreover, it was recently demonstrated by Wong et al. (Wong,
Anzola et al. 2015) that cancer cells are fundamentally different from normal cells in their response to Plk4 inhibition and subsequent centriole loss. While centrosome loss irreversibly arrests normal cells in G1 by a p53-dependent mechanism, cancer cells can continue to proliferate indefinitely, highlighting the importance of this line of investigation. Plk4 depletion using shRNA or siRNA delayed migration and/or invasion in HeLa (cervical), MDA-MB-435 (breast/melanoma) and MDA-MB-231 (breast) cancer cells, while Plk4 upregulation using a stable inducible system in HeLa and U2OS (osteosarcoma) cell lines promoted migration and/or invasion. The highly selective inhibitor of Plk4 kinase activity centrinone B also suppressed wound healing by MDA-MB-231 cells. Taken together, these results implicate Plk4 in regulating cancer cell migration and invasion, suggesting a role in tumour progression. Nevertheless, the precise mechanisms/pathways or substrates mediating Plk4’s effect on migration and invasion remained unknown and became the focus for the following chapter.
Chapter 4: Plk4 regulates invasion and metastasis in vivo

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


4.1 Overview

While the critical role played by Plk4 in centriolar biogenesis is well-acknowledged, the localization of Plk4 at the edges of lamellipodia and filopodia we found in motile cells had been unexpected, as was the correlation between Plk4 expression and motility in MEFs. The studies described in Chapter 3 showed that Plk4 regulates cancer cell spreading, polarity, migration and invasion in vitro. The results described in this chapter show that Plk4 also promotes cancer progression in vivo; I demonstrate that the invasive and metastatic progression of the human breast cancer line MDA-MB-231 in murine xenografts is dependent on Plk4 expression. Stable knockdown of Plk4 in MDA-MB-231 xenografts resulted in a modest suppression of tumour growth. Histological analysis at early timepoints (4-6 weeks, tumours matched for size) showed a marked inhibition of invasion into underlying muscle and lymphovascular invasion in Plk4 shRNA xenografts. At later timepoints (7-10 weeks, tumours matched for size), Plk4 knockdown similarly inhibited gross invasion through the abdominal wall and into the peritoneal cavity. In addition, lung metastasis was markedly suppressed in Plk4 shRNA xenograft mice, with reduced incidence of metastatic colonies and much smaller size of micro-metastases. This was in conjunction with a gene expression signature consistent with mesenchymal-to-epithelial reprogramming in cancer cells upon Plk4 knockdown. These data fit with my hypothesis that increased Plk4 levels in established malignancy may promote tumor progression. Indeed, high
Plk4 levels have been reported in many tumours, including breast and colorectal cancers (Macmillan, Hudson et al. 2001, Mason, Lin et al. 2014). The dependence of cancer invasion and metastasis on Plk4 in preclinical models that I have shown here supports the evaluation of Plk4 inhibitors in patients who experience cancer progression on conventional chemotherapy.
4.2 Introduction

While elderly Plk4\textsuperscript{+/−} mice have a 50% incidence of spontaneous tumour formation, the majority being multifocal hepatocellular carcinomas (Ko, Rosario et al. 2005), high levels of Plk4 expression have been reported in many solid tumour types, including breast and colorectal cancers, glioblastoma, and bladder cancer (Macmillan, Hudson et al. 2001, Mason 2011, Mason, Lin et al. 2014). Initial studies to explore Plk4 overexpression and tumourigenesis in \textit{Drosophila} showed that elevated expression of Plk4 in larval brain cells, when transplanted into the abdomens of wildtype adult flies can generate metastatic tumours (Basto, Brunk et al. 2008). Recently published work in transgenic mice with Plk4 overexpression, while contradictory, has now described accelerated tumour formation in a p53 null background (Coelho, Bury et al. 2015, Kulukian, Holland et al. 2015, Vitre, Holland et al. 2015, Sercin, Larsimont et al. 2016), although this was not known at the beginning of my studies. Emerging evidence from our own laboratory suggested that increased Plk4 levels in established malignancy might promote tumour progression by increasing cancer cell motility and invasion \textit{in vitro} (see Chapter 3; published in part in (Rosario, Kazazian et al. 2014)). In analogous experiments, Godhino et al. (Godinho, Picone et al. 2014) used a 3-D culture system to show that Plk4 overexpression triggers mammary epithelial cell invasion, an effect they attributed to centrosome amplification. In breast cancer specifically, Plk4 has been identified as an element of a “stem cell like” expression signature that is not only associated with resistance to therapy but also death from metastases (Glinsky 2006, Finetti, Cervera et al. 2008, Agarwal, Gonzalez-Angulo et al. 2009, Mason, Lin et al. 2014), further suggesting a relationship between Plk4 and tumour progression. Denu et al. (Denu, Zasadil et al. 2016) showed that in breast cancer, centrosome amplification is associated with reduced overall survival and recurrence-free survival, and correlates strongly with high-risk
subtypes (triple negative and HER2-amplified) and higher stage and grade. Mak and colleagues had previously shown a correlation between increased Plk4 expression and the same clinically high risk features (Mason 2011). Forced expression of Plk4 in breast cancer cells led to high-grade features with cells that were more de-differentiated (Denu, Zasadil et al. 2016), which the authors attributed to the centrosome amplification induced by Plk4.

As will be shown in Chapter 5, my work has indicated an alternate mechanism whereby Plk4 expression drives cancer progression – via enhanced cancer cell migration and invasion due to altered actin rearrangement dynamics. When considering the potential role of Plk4 in cancer progression through this mechanism, the complexity of cell movement and its regulation must be recognized. In some cases, invasion in vitro does not correlate with invasion and metastatic capacity in vivo (Schaeffer, Somarelli et al. 2014), which can be due to the modulating effects of the tumour microenvironment on cancer cell behaviour and requirement for breakdown of extracellular matrix to invade surrounding stroma. Therefore, while in vitro models are frequently used to assess cell migration/invasion, they are sometimes considered to have low physiological relevance. The goal of this section was therefore to explore the effect of Plk4 on in vivo invasion and metastasis, which has not to date been assessed by other research groups.
4.3 Results

*Plk4 facilitates invasion and metastasis in vivo.*

We used a flank xenograft model with MDA-MB-231 breast cancer cell lines transduced with RFP or Plk4 shRNAs (#1 and #2) in NOD SCID mice to investigate the effect of Plk4 on tumour progression *in vivo*. MDA-MB-231 cells were chosen for several reasons: they have been previously reported to grow highly invasive tumours when injected subcutaneously into immunocompromised mice; they have moderately high endogenous Plk4 expression (Mason, Lin et al. 2014); and Plk4 depletion robustly impaired migration and invasion in *in vitro* assays without altering proliferation (Figure 3.6). The flank injection site was chosen as it allowed for assessment of local invasion into underlying flank muscle, as well as spontaneous metastasis. The effect of Plk4 shRNA transduction on Plk4 expression and invasion was confirmed to be stable over long term culture of cells prior to initiation of the studies in mice (Figure 4.1a).

Initial trials of subcutaneous injection of MDA-MB-231 cells into NCr Nude mice did not generate flank tumours, while palpable tumours developed in NOD SCID mice after 2-3 weeks. A pilot experiment established that injection of 1x10⁶ cells in Matrigel had an optimal time line of growth for subsequent experiments on Plk4 depletion, which was expected to slow growth based on the experience reported by Mason et al. (2014). Tumour growth was in fact mildly suppressed in Plk4-depleted versus RFP shRNA control tumours, beginning at approximately 5 weeks after subcutaneous injection (Fig. 4.1b). Therefore, a timepoint for sacrifice was established centered around 5 weeks (range 4-6 weeks; Table 4.1) when tumours had achieved a threshold volume. I did attempt to harvest all tumours at 4 weeks, when there was no difference in tumour size, however the tumours were difficult to handle due to their small size, had a large
Figure 4.1

(a) Relative Plk4 mRNA expression (ΔΔCt) over time (days).

(b) Tumor volume (mm^3) over time (weeks).

(c) Relative Plk4 mRNA expression (ΔΔCt) for different time periods.

(d) Immunohistochemistry showing ki67 expression.

(e) Percent ki67 positive cells over time.
**Figure 4.1. Plk4 knockdown suppresses MDA-MB-231 flank xenograft growth.** a) Relative Plk4 mRNA levels in MDA-MB-231 cells transduced with RFP or Plk4 shRNAs, showing persistent reduction in Plk4 expression over time in culture. GAPDH used for internal control. 

(b-e) MDA-MB-231 cells were injected subcutaneously into the right flanks of NOD SCID mice and followed over time. b) Tumour volume of xenografts calculated assuming an ellipsoid shape, showing modest growth suppression in Plk4 vs. RFP shRNA xenografts after 5 weeks, *p<0.01 vs. Plk4 shRNAs, analyzed using Anova with Bonferroni correction at each timepoint. c) Relative quantification of Plk4 mRNA levels in tumours at 4-6 weeks and 7-10 weeks after injection, showing persistence of Plk4 depletion, *p<0.0001 vs. RFP shRNA. d) Representative images of formalin fixed paraffin-embedded sections from the indicated flank tumours stained using hematoxylin & eosin (H&E), or immunostained for Ki67. Bars: 100 µm. e) Quantification of percent Ki67 positive cells in tumour sections, p>0.6, using ImageJ image analysis software.
Table 4.1 Time to sacrifice post subcutaneous RFP or Plk4 shRNA MDA-MB-231 injection.

<table>
<thead>
<tr>
<th>Early tumours: Time of sacrifice (weeks post-injection)</th>
<th>RFP shRNA</th>
<th>Plk4 shRNA#1</th>
<th>Plk4 shRNA#2</th>
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<tr>
<td>Median (Range)</td>
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<td>5.25 (5-6)</td>
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<th>Plk4 shRNA#2</th>
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<tr>
<td>Median (Range)</td>
<td>8 (7-9)</td>
<td>8.5 (7.5-9.5)</td>
<td>9 (8-10)</td>
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Matrigel component, and there was frequently not enough tumour tissue for downstream analysis. Because size would presumably confound the assessment of local invasion and metastasis, we made the decision to size match tumours for analysis. We chose a threshold tumour size to prompt for sacrifice, which was therefore performed over a range of time points (4-6 weeks for the “early” analysis). Mice were also followed to later timepoints to assess for development of spontaneous metastases. As fifty percent of RFP shRNA mice had reached endpoints defined by the TCP Animal Care Committee by 8-9 weeks post-injection, the average tumour volume of RFP shRNA xenografts at ~7 weeks was used to determine a second threshold volume for sacrifice to assess for metastatic disease (Table 4.1). In all MDA-MB-231 xenografts, I confirmed that Plk4 expression in vivo was reduced to 25-45% of RFP control, and this reduction was durable up to at least 10 weeks, for each of the two Plk4 shRNA constructs (Figure 4.1c). Size matching based on the flank tumour required that we delay sacrifice of Plk4 shRNA mice to approximately 9-10 weeks; these animals were assessed for number and size of lung metastases, as well as gross depth of invasion of the flank tumour itself.

Despite the mild suppression of tumour growth with Plk4 depletion, immunohistochemical staining for Ki67 revealed similar rates of cellular proliferation in Plk4-depleted vs. RFP control tumours (Figure 4.1d,e). H&E staining of the size-matched (Figure 4.3b) flank tumour nodules at 4-6 weeks after flank injection showed the presence of cancer invadopods reaching into the underlying skeletal muscle in RFP control but not Plk4 shRNA tumours (Figure 4.2a). Other signs of invasion on histology included lymphovascular invasion or the presence of satellite tumour nodules adjacent to the main tumour mass in RFP shRNA xenografts, with one sole Plk4 shRNA tumour displaying lymphovascular invasion. Tumours were harvested with overlying skin and underlying flank tissue, and split with half the tumour snap frozen in liquid nitrogen for
Figure 4.2

(a) Histological images showing the effects of RFP shRNA and Plk4 shRNA on tumor development.

(b) Images depicting the effects on metastatic colony area and number of metastatic colonies per lung.

(c) Immunohistochemical staining for vimentin.

(d) Statistical graphs comparing metastatic colony area and number of metastatic colonies across treatment groups.
Figure 4.2. Plk4 facilitates MDA-MB-231 xenograft invasion and metastasis in vivo.  
a) Representative images of H&E or pan-Cytokeratin (PCK) immunohistochemical staining on 4-6-week tumour sections showing invasion into underlying muscle in RFP shRNA tumours (top panels), but not Plk4 shRNA tumours.  
b) Representative images of flank tumours 7-10 weeks post-injection, showing gross invasion into the peritoneum in RFP shRNA controls and no invasion in Plk4 shRNA tumours.  
c) Representative images of metastatic deposits in lungs of mice harboring the indicated MDA-MB-231 flank xenografts, stained using H&E and immunostained for vimentin. Bars: 100µm.  
d) Number (top panel) and area (bottom panel) of metastatic colonies in lungs of mice harboring MDA-MB-231 RFP or Plk4 shRNA tumours, *p < 0.001 vs. RFP shRNA. For panels a-d, the Plk4 shRNA flank tumours were analyzed when they reached a comparable size to that of RFP shRNA tumors and the metastases in Plk4 shRNA mice were analyzed when the flank tumors reached a comparable size to that of RFP shRNA mice. Data are means±SEM.
real time-PCR, and the remaining tumour fixed for histology. Four sections of tumour 200µm apart were stained with H&E and evaluated in a blinded manner by pathologist Dr. Hannah Wu for invasion. When harvesting the tumours, the characteristics of the tissues were perceptibly different between Plk4 and RFP shRNA xenografts; Plk4 shRNA xenografts were very mobile in relationship to the underlying tissues and care had to be taken not to “lift off” the tumour from the underlying flank, while RFP shRNA xenografts were more fixed. The difference in invasive capacity between Plk4 and RFP shRNA xenografts became grossly apparent by 7-10 weeks, at which point RFP control xenografts had penetrated into the peritoneal cavity and caused peritoneal carcinomatosis, whereas the majority of Plk4-depleted tumour nodules remained superficial to the abdominal wall musculature (Figure 4.2b). Intraabdominal metastases occurred in only the control xenografts, and included a single large liver metastasis, ovarian metastases and seeding of tumour on the stomach, large and small intestines and the liver capsule (n=6 mice). Thus while the effect of Plk4 on primary tumour growth appeared relatively minor, at least for the level of depletion achieved here, its effect on invasion into surrounding tissues was more profound.

Dr. Wu also carefully examined the lungs of mice with size-matched primary tumours (Figure 4.3b) at the time of sacrifice (7-9 weeks post injection for RFP controls, 8-10 weeks for shPlk4), using both H&E and vimentin staining to detect, enumerate and measure metastases. Lung metastases were significantly more numerous and larger in size in the controls than in the mice with Plk4 depleted tumours (Figure 4.2c,d).
**Figure 4.3**

### a

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<th></th>
<th>4-6 weeks</th>
<th>7-10 weeks</th>
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<tbody>
<tr>
<td></td>
<td>Invasion into muscle</td>
<td>Lymphovascular Invasion</td>
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<tr>
<td>RFP shRNA</td>
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<td>6 / 14</td>
</tr>
<tr>
<td>Plk4 shRNA#1</td>
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<tr>
<td>Plk4 shRNA#2</td>
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**RFP**

**Plk4**

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<th>4-6 weeks</th>
<th>7-10 weeks</th>
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<tr>
<td></td>
<td>Tumour volume (mm$^3$)</td>
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<td>RFP shRNA</td>
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<td><img src="image2" alt="Graph" /></td>
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<td>Plk4 shRNA#1</td>
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<td>Plk4 shRNA#2</td>
<td><img src="image5" alt="Graph" /></td>
<td><img src="image6" alt="Graph" /></td>
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**b**

4-6 weeks

7-10 weeks

**c**

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<tr>
<th></th>
<th>Relative Plk1 mRNA expression (ΔΔCt)</th>
<th>Relative Plk2 mRNA expression (ΔΔCt)</th>
<th>Relative Plk3 mRNA expression (ΔΔCt)</th>
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<tr>
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<tr>
<td>Plk4 shRNA</td>
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Figure 4.3. Summary of Plk4-mediated xenograft invasion and metastasis.  

a) Summary of invasive and metastatic phenotypes in RFP and Plk4 shRNA MDA-MB-231 xenograft mice. 4 independent experiments, total number of mice per group as shown.  
b) Mice were sacrificed at an appropriate timepoint based on attainment of threshold tumour volume. Note that tumour volumes were not significantly different between Plk4 and RFP shRNA xenografts (p=0.08 at 4-6 weeks; p=0.162 at 7-10 weeks).  
c) Relative Plk1, Plk2 or Plk3 mRNA levels in MDA-MB-231 xenografts. Left panel, p=0.8, n=10; Middle panel, p=0.7, n=6; Right panel, p=0.97, n=26. RPII used as internal control. Data are means±SEM.
Overall, at 4-6 weeks post injection, we noted evidence of locally aggressive behaviour in 11 of 14 (79%) controls versus only 1 of 16 (6%) Plk4 shRNA primary tumours (Figure 4.3a). At 7 - 10 weeks, peritoneal invasion and lung metastases were seen in 89% and 100% of control mice, but only 7% and 29%, respectively, of Plk4-depleted xenograft mice (Figure 4.3a). Of note, the expression of Plks1-3 in the Plk4-depleted xenografts was comparable to RFP controls (Figure 4.3c).

We had noted a pronounced alteration in the morphology of cultured HeLa cells stably depleted of Plk4, which appeared less fibroblastic and more clustered than control cells, consistent with a shift from a classic mesenchymal to a more epithelial phenotype (Figure 4.4a). Indeed, Plk4-depleted cells gained expression of the epithelial marker E-cadherin, at both protein and mRNA levels, and lost expression of fibronectin and other mesenchymal markers (Figure 4.4a). As with cells growing in culture, Plk4-depleted tumours displayed a more "epithelial" gene expression pattern than did control tumours (Fig. 4.4b).
Figure 4.4

a

- Luciferase shRNA
- Plk4 shRNA #1
- Plk4 shRNA #2

Relative mRNA expression (ΔΔCt)

b

- E-cadherin
- Fibronectin
- MMP-2
- N-cadherin

Relative mRNA expression (ΔΔCt)
Figure 4.4. Plk4 promotes a mesenchymal phenotype and expression profile. a) Left panels, representative bright-field images showing cell morphology in Plk4 shRNA stable HeLa cell lines. Middle panel, immunoblots for E-cadherin, Fibronectin or γ-tubulin on lysates, as above (representative of three independent experiments). Relative mRNA levels for genes associated with mesenchymal to epithelial transition (right panel), show development of an epithelial expression profile when Plk4 is depleted. GAPDH used for internal control, n=3, *p<0.05 vs. Luciferase shRNA. b) Relative mRNA levels in the indicated MDA-MB-231 flank xenografts for genes associated with mesenchymal to epithelial transition. RPII used for internal control, *p<0.05, **p<0.01 vs. RFP shRNA tumours. Data are means±SEM.
4.4 Discussion

Our understanding of the role of Plk4 in mammalian cancer development and progression has been hampered by its precise self-regulation; Plk4 dimerizes and phosphorylates itself to promote its own destruction and Plk4 protein is thereby of low-abundance in cells. Dimerization and autophosphorylation are also required for the kinase activity that activates downstream substrates, making the relationship between Plk4 protein abundance and activity complex (Rogers, Rusan et al. 2009, Guderian, Westendorf et al. 2010, Sillibourne, Tack et al. 2010). Moreover, cell migration/invasion is a highly multifaceted process with numerous inputs and levels of regulation. I have shown that Plk4 promotes migration and invasion in vitro in several cancer cell lines. However, in some cases, invasion in vitro does not correlate with invasion and metastatic capacity in vivo (Schaeffer, Somarelli et al. 2014), which can be due to the modulating effects of the tumour microenvironment. The results described in this chapter show that Plk4 does in fact promote cancer cell invasion and metastatic progression in vivo, and these findings deepen our appreciation of Plk4 as a potential therapeutic target in cancer treatment.

In the MDA-MB-231 breast cancer xenograft model we used, the effect of depletion of Plk4 to 25-30% of control levels had a modest effect on tumour growth, with no difference in expression of the proliferation marker Ki67. We had similarly observed no effect on the proliferation of cancer cells in culture with Plk4 depletion (Chapter 3). These observations are consistent with the recent demonstration by Wong et al. that cancer cells are fundamentally different from nontransformed cells in their tolerance of an acentrosomal state, where the highly selective and effective Plk4 inhibitor centrinone was able to block centriole duplication, and while this led to arrest and death of normal cells, cancer cells continued to proliferate, albeit at a reduced rate (Wong, Anzola et al. 2015). This presents an important caveat for the application of Plk4
inhibitors in cancer therapy, where greater utility may be found with the goal of suppression of invasion and metastasis, as our data indicate. Yet in some systems, modest depletion of Plk4 has been shown to significantly decrease proliferation. Mason et al. showed that transduction of MDA-MB-468 cells with Plk4 shRNA and reduction of Plk4 transcript levels in these cells to approximately 40% of control resulted in a significant decrease in in vivo growth, although the level of depletion in the xenografts was not reported (Mason, Lin et al. 2014). The differences observed between the work presented in this Chapter and the study by Mason et al. may be due to the level of depletion achieved in each model, the endogenous Plk4 expression in the cancer cell lines (MDA-MB-468 cells have approximately half the Plk4 mRNA levels of MDA-MB-231 cells), or alternatively the PTEN status of the cells: MDA-MB-468 cells are PTEN deficient while MDA-MB-231 cells are PTEN wildtype. A synthetic lethal interaction between Plk4 and PTEN has been reported in breast cancer cells (Brough, Frankum et al. 2011), and CFI-400945 has increased in vivo activity in reducing xenograft size in PTEN null relative to PTEN wild-type tumour models (Mason, Lin et al. 2014). PTEN may therefore be an important biomarker for consideration in the application of Plk4 inhibitors in patients.

To attempt to control for the effect of differential tumour growth on invasion and metastasis phenotypes, mice were sacrificed when the tumours achieved a threshold volume. Two timepoints were chosen for analysis, between 4-6 weeks to evaluate for microscopic invasion phenotypes including presence of invadopods extending into underlying muscle, lymphovascular invasion, and satellite tumours, and 7-10 weeks to evaluate for macroscopic invasion through the flank musculature and into the peritoneum and for the presence of lung micrometastasis. At both timepoint ranges, all attempts were made to match the xenografts for size, and therefore Plk4 shRNA xenograft mice were commonly sacrificed at later times after flank injection compared to
controls (Table 4.1). Nevertheless, Plk4 depletion resulted in less locally aggressive behaviour and less metastatic progression than controls. Of note, Plk1 has also been described as an oncogene, while Plks 2 and 3 have tumour suppressive functions, and in our model xenograft lystaes did not show altered expression of Plks1-3 in the Plk4-depleted xenografts compared to controls, showing specificity of the invasive and metastatic phenotypes to Plk4.

Further, in our analysis of the phenotypic features of Plk4 depletion in HeLa cancer cells in culture, we noted a marked change in the morphology of the cells when depleted of Plk4 with shRNA, which appeared less fibroblastic and more clustered than control cells, reminiscent of a shift from mesenchymal to epithelial phenotype. Induction of EMT is considered by some a key step in cancer metastasis and progression (Thiery 2002). Metastatic carcinoma progression consists of several distinct steps, including acquisition of the ability of epithelial tumour cells to invade through the basement membrane by degradation of the extracellular matrix (ECM), intravasation into the circulatory or lymphatic systems, extravasation into a distant site, followed by proliferation. The EMT program describes the process whereby the epithelial cells lose their epithelial characteristics, such as cobblestone-like morphology, tight cell-cell junctions, apical-basal polarity and cell-to-cell adhesion, and undergo dramatic remodelling of the cytoskeleton (Thiery 2002). Concurrently, cells undergoing EMT acquire expression of mesenchymal components and a migratory phenotype, with evidence indicating that cancer cells activate the EMT program, traditionally described in embryogenesis, in promoting cell migration and increasing the metastatic and invasive potential of these cells (Kang and Massague 2004, Yang, Mani et al. 2004, Huber, Kraut et al. 2005, Guarino 2007, Tiwari, Gheldof et al. 2012). Downregulation of epithelial markers such as E-cadherin, and upregulation of mesenchymal markers such as fibronectin, vimentin and N-cadherin characterize the EMT process. Indeed, the
most important event in classic epithelial to mesenchymal transition is loss of E-cadherin (CDH1), which has been shown to be a prerequisite for epithelial tumour cell invasion. Here we show that HeLa cells, which express Twist and thereby have a program for EMT in place, when depleted of Plk4 gained expression of the epithelial marker E-cadherin, and lost expression of fibronectin and other mesenchymal markers. Plk4-depleted MDA-MB-231 xenografts also displayed a more "epithelial" gene expression pattern than did control tumours in the majority of genes assayed, in keeping with the phenotype of decreased tumour invasiveness. However, how EMT is induced during Plk4-related cancer progression remains unexplored. Several signalling pathways are implicated in contributing to EMT, including TGF-β, receptor tyrosine kinase/Ras, and Wnt/Notch-dependent signaling, as are transcriptional regulators such as Snail, Snail2/Slug, and Twist which have a regulatory role in the mechanism of EMT mainly through the repression of E-cadherin (Cano, Perez-Moreno et al. 2000, Huber, Kraut et al. 2005). Of the transcription factors capable of repressing E-cadherin, we do show reduced expression of Snail2/Slug with Plk4 depletion. In addition, the relationship between Plk4 and p53 expression, where both Plk4 mediated centriole loss and amplification induce p53 expression, and p53 in turn represses EMT by activating expression of various microRNAs to maintain an epithelial phenotype (Chang, Chao et al. 2011), are potential mechanisms that can be explored in future experiments.

Recent publications have now described a role for Plk4 overexpression in tumourigenesis in p53 null mice. While some studies have not shown increased tumour formation in Plk4 overexpressing transgenic mice (Kulukian, Holland et al. 2015, Vitre, Holland et al. 2015), Coelho and colleagues utilized a Cre-recombination approach to generate transgenic mice with doxycycline inducible expression of Plk4 in a p53 null background and found accelerated tumour formation, namely lymphomas and sarcomas in the Plk4 overexpressing mice (Coelho, Bury et
al. 2015). Sercin and colleagues (Sercin, Larsimon et al. 2016) similarly developed a genetic mouse model conditionally overexpressing mCherry-Plk4 with K14Cre, allowing for K14Cre mediated recombination and overexpression of mCherry–Plk4 during mouse skin epidermis development. Again, p53 deletion combined with Plk4 overexpression resulted in the generation of aneuploidy and triggered the formation of squamous cell carcinomas with complete penetrance; while control and Plk4-overexpressing/p53 intact mice did not develop spontaneous skin tumours, all Plk4 overexpressing/p53KO mice developed spontaneous tumours with a shorter latency and an increased number of tumours per mouse as compared to p53KO mice (Sercin, Larsimont et al. 2016). In light of these conflicting findings, it will certainly be interesting to follow upcoming research in the Plk4 field in relation to tumourigenesis.
Chapter 5: Plk4 interacts with Arp2 to promote cancer cell migration

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


5.1 Overview

Metastasis remains the most common cause of death following resection of primary melanoma, breast, pancreas, prostate and colorectal cancers. High expression of Plk4 in several of these cancers predicts aggressive behaviour and resistance to therapy, suggesting a role in cancer progression. My objective was to describe and understand the mechanism(s) of Plk4’s oncogenic effect, with the eventual goal of therapeutically modulating the pathways/networks that facilitate metastatic capacity. The stimulatory effect of Plk4 on mammary gland invasiveness in vitro was previously attributed to activation of Rac1 by centrosome amplification (Godinho, Picone et al. 2014). To query the potential relationship between centriole amplification and altered motility, I assessed the effects of Plk4 over-expression or knockdown on centriole number and motility in several cell lines. While the direction of the change in centriole number generally correlated with the direction of change in motility, the magnitude of the changes was often disproportionate. I also interrogated the role of Rac1 in mediating the enhancement of cancer cell migration by Plk4. While dominant negative Rac1T17N suppressed wound healing in the absence of tet-induced Plk4, stimulation of U2OS migration by Plk4 was not affected by the presence of Rac1T17N, suggesting potential mediation by an additional mechanism. Directional cell polarity was impaired by Plk4 knockdown, and actin rearrangement towards a scratch wound was markedly reduced. Investigation of pathways upstream of actin polymerization revealed that Plk4 regulates...
activation of Cdc42, although Cdc42T17N had no effect on directional migration in U2OS cells. Interaction proteomics identified members of the Actin Related Protein (Arp) 2/3 complex as novel Plk4 interactors, and I confirmed a physical and functional interaction between Plk4 and the Arp2/3 complex member Arp2 in mediating Plk4-driven cancer cell movement. This interaction is mediated through the Plk4 PB1-PB2 domain, and results in phosphorylation of Arp2 at the T237/T238 activation site. Our results validate Plk4 as a therapeutic target in cancer patients, and reveal a new role for Plk4 in regulating Arp2/3-mediated actin cytoskeletal rearrangement.
5.2 Introduction

Disturbances in multiple cellular mechanisms are implicated in carcinogenesis, including centriole duplication, cell motility/invasion, and mitotic progression. Prevention and control of tumour invasion has become a major therapeutic focus as death from solid tumours is most commonly related to invasion and metastases. In chapter 3, I described the effect of Plk4 on MEF and cancer cell spreading, migration and invasion, and in chapter 4 showed the promotion of cancer invasion by Plk4 is relevant in vivo; however, the mechanism(s) by which Plk4 is enhancing cell motility and invasion were largely unexplored.

Our understanding of the role of Plk4 in mammalian cancer development and progression has been hampered by its precise autoregulation and resultant paucity at the protein level. Despite demonstration of a consensus sequence for phosphorylation by the Plk4 kinase domain, Plk4 is a serine-threonine kinase for which relatively few bona fide functional substrates had been identified. Plk4’s best characterized substrate is Plk4 itself, with autophosphorylation occurring in trans at multiple residues in the phosphodegron. Dimerization and autophosphorylation are required for Plk4 kinase activity to activate downstream targets while simultaneously triggering ubiquitination and proteosomal degradation of Plk4 itself (Guderian, Westendorf et al. 2010, Holland, Lan et al. 2010, Cunha-Ferreira, Bento et al. 2013, Klebba, Buster et al. 2013), making the relationship between kinase activity and protein level a particularly complex one. GCP6, a core component of the γ-tubulin ring complex, is phosphorylated by Plk4 to regulate centriole duplication (Bahtz, Seidler et al. 2012), as is FBXW5 thereby suppressing its ability to ubiquitinate Sas-6 to restrict centriole duplication (Puklowski, Homsi et al. 2011). Plk4 has also been shown by one group to phosphorylate CEP152, although others did not find evidence of CEP152 phosphorylation by Plk4. CEP152 is generally considered a scaffolding protein that
targets Plk4 to the centriole (Cizmecioglu, Arnold et al. 2010, Dzhindzhev, Yu et al. 2010, Hatch, Kulukian et al. 2010). In the established centriole duplication pathway in human cells, CEP152 and CEP192 cooperate to recruit Plk4 to the proximal end of the mother centriole to initiate centriole duplication at the G1/S transition (Kim, Park et al. 2013, Sonnen, Gabryjonczyk et al. 2013). Subsequently Plk4 phosphorylates the key centriolar protein STIL to recruit STIL to the centriole, and prime direct binding of STIL to Sas-6 to trigger cartwheel formation (Kratz, Barenz et al. 2015, Moyer, Clutario et al. 2015). Identification of STIL as a key centriolar Plk4 substrate in recent years provided the first link between Plk4 catalytic kinase activity and centriole duplication (Dzhindzhev, Tzolovsky et al. 2014, Ohta, Ashikawa et al. 2014).

Known extra-centrosomal substrates for Plk4 are limited to Hand1 and Ect2. Plk4-mediated phosphorylation and activation of the bHLH transcription factor Hand1 is necessary for trophoblast differentiation (Martindill, Risebro et al. 2007). Our group has also shown that Plk4 phosphorylates the RhoGTPase GEF Ect2, and that activation of RhoA upon Plk4 transfection is Ect2-dependent. Furthermore, haploid levels of Plk4 are associated with a defect in cytokinesis related to failure to appropriately activate RhoA in mitotic cells (Rosario, Ko et al. 2010). While RhoA functions in cell cycle progression, another key role for RhoA is in cell migration. In the protrusions of HeLa cells co-transfected with Flag-Plk4 and GFP-Ect2, we observed Ect2 and Plk4 staining in a similar distribution, suggesting that activation of RhoA in cell protrusions by Plk4 may be effected through its interaction with Ect2 (Rosario, Kazazian et al. 2015). Plk4 overexpression and centrosomal amplification have also been shown by Pellman’s group to activate the RhoGTPase Rac1 to facilitate cell invasion in vitro (Godinho, Picone et al. 2014). Godhino et al. (2014) showed that centrosome amplification, whether caused by Plk4 overexpression or cytokinesis failure, triggers the formation of invasive protrusions in 3D
cultures, which they attributed to inappropriate activation of Rac1, disrupting normal cell-cell adhesion. The RhoGTPase Cdc42, the key regulator of polarity, however, had not previously been shown to be affected by Plk4.

In the previous chapters I showed that Plk4 promotes cancer cell spreading, protrusion formation, migration and invasion. However, Plk4 substrates involved in cell motility and invasion had not been identified at the outset of these studies. The goal of the experiments described in this chapter was to uncover the pathways/mechanisms by which Plk4 affects cancer cell migration and invasion, with the goal of suppressing Plk4-mediated tumour progression to improve survival in patients with aggressive tumours.
5.3 Results

5.3.1 Plk4-induced change in migration and invasion and magnitude of centriole amplification or depletion

As increased invasiveness has been ascribed to centrosome amplification by other groups (Godinho, Picone et al. 2014, Denu, Zasadil et al. 2016), I investigated the effect of Plk4 manipulation on centriole number for each cell line and treatment condition I had used to study the effect of Plk4 expression on cancer cell migration and invasion in Chapter 3 (Figure 5.1, Figure 3.7c,d; Figure 3.8c,d). A summary is presented in Table 5.1. As expected, Plk4 depletion using shRNA or siRNA decreased Plk4 expression and corresponded to an increase in the proportion of cells with \( \leq 1 \) centriole, while Plk4 overexpression using a tetracycline inducible system in U2OS and HeLa cells resulted in increased Plk4 expression and an increase in the proportion of cells with \( \geq 5 \) centrioles. With the exception of Plk4\(^{+/-}\) MEFs, which display centrosome amplification, the direction of the change in centriole number correlated with the direction of change in motility, as illustrated for migration (Table 5.1). However, the magnitude of the effects were often discrepant. For instance, while stable upregulation of Plk4 in HeLa or U2OS cells differentially increased centriole number in each of the two cell lines, the stimulatory effect on migration was not correspondent (Figures 3.7; Figure 3.8). In U2OS cells, induction of Plk4 expression using 0.01\( \mu \)g/ml tetracycline had minimal impact on centriole number, but significantly increased wound healing. There were similar discrepancies in the magnitude of the effects of Plk4 manipulation on centriole number and invasion (Figure 3.9; Figure 5.1), suggesting the possibility that the effect of Plk4 on cell motility may be mediated, at least in part, through a mechanism other than loss or gain of centrioles.
MDA-MB-231

**Figure 5.1**

a. Graph showing relative Plk4 mRNA expression (ΔΔCt) for RFP shRNA, Plk4 shRNA#1, and Plk4 shRNA#2.

b. Images of MDA-MB-231 cells showing centrin-positive foci for RFP shRNA, Plk4 shRNA#1, and Plk4 shRNA#2.

c. Bar graph showing percent cells (centrin-positive foci) for RFP shRNA, Plk4 shRNA#1, and Plk4 shRNA#2.

HeLa 48h siRNA

d. Graph showing relative Plk4 mRNA expression (ΔΔCt) for Luciferase siRNA, Plk4 siRNA-pool, Plk4 siRNA-A, Plk4 siRNA-B, and Plk4 siRNA-C.

e. Images of HeLa cells showing centrin-positive foci for Luciferase siRNA, Plk4 siRNA-A, Plk4 siRNA-B, and Plk4 siRNA-C.

f. Bar graph showing percent cells (centrin-positive foci) for Luciferase siRNA, Plk4 siRNA-A, Plk4 siRNA-B, and Plk4 siRNA-C.

HeLa

g. Graph showing relative Plk4 mRNA expression (ΔΔCt) for Luciferase shRNA, Plk4 shRNA#1, and Plk4 shRNA#2.

h. Images of HeLa cells showing centrin-positive foci for Luciferase shRNA, Plk4 shRNA#1, and Plk4 shRNA#2.

i. Bar graph showing percent cells (centrin-positive foci) for Luciferase shRNA, Plk4 shRNA#1, and Plk4 shRNA#2.

MDA-MB-435

j. Graph showing relative Plk4 mRNA expression (ΔΔCt) for Luciferase shRNA, Plk4 shRNA#1, and Plk4 shRNA#2.

k. Images of MDA-MB-435 cells showing centrin-positive foci for Luciferase shRNA, Plk4 shRNA#1, and Plk4 shRNA#2.

l. Bar graph showing percent cells (centrin-positive foci) for Luciferase shRNA, Plk4 shRNA#1, and Plk4 shRNA#2.
Figure 5.1. Plk4 knockdown in MDA-MB-231, HeLa and MDA-MB-435 cancer cell lines.

a) Relative Plk4 mRNA levels in MDA-MB-231 cells transduced with RFP or Plk4 shRNAs, n=3, *p<0.0001 vs. RFP shRNA. b) Representative immunofluorescence images of MDA-MB-231 RFP or Plk4 shRNA cells labeled with antibodies to centrin (red), pericentrin (green) and Hoechst (blue). Percent of cells with illustrated phenotype is shown. The right panels/inserts show magnified centrosomes (boxed in white) for each condition. c) Bar graph showing frequency of cells with indicated number of centrin-positive foci in cells as in b. d) Relative Plk4 mRNA levels in HeLa cells transfected with Luciferase or Plk4 siRNAs for 48h, n=2, *p<0.0005 vs. Luciferase siRNA. Bottom panel, representative immunofluorescence images of HeLa cells transfected as above and labeled with antibodies to pericentrin (red), Plk4 (green) and Hoechst (blue). e) Representative immunofluorescence images of HeLa cells transfected with the indicated siRNAs labeled as in b. f) Bar graph showing indicated number of centrin-positive foci in cells as in e. g) Relative Plk4 mRNA levels in HeLa stable cell lines transduced with Luciferase or Plk4 shRNAs, n=2, *p=0.002 vs. Luciferase shRNA. h) Representative immunofluorescence images of HeLa Luciferase or Plk4 shRNA cells labeled as in b. i) Bar graph showing indicated number of centrin-positive foci in cells as in h. j) Relative Plk4 mRNA levels in MDA-MB-435 cells transfected with Luciferase or Plk4 shRNAs, n=3, *p<0.001 vs. Luciferase shRNA. k) Representative immunofluorescence images of MDA-MB-435 Luciferase or Plk4 shRNA cells labeled as in b. l) Bar graph showing indicated number of centrin-positive foci in cells as in k. c,f,i,l: n>60 cells, 2 independent experiments. For real time-RT PCR, GAPDH mRNA was used for normalization. Bars: 10µm, insert 1µm. Data are means±SEM.
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Δ Plk4 expression* (% of control)</th>
<th>Effect on centrioles* (centriole scale)</th>
<th>Δ Migration* (% of control)</th>
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<tbody>
<tr>
<td>MDA-MB-231 Plk4 shRNA</td>
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<tr>
<td>HeLa Plk4 siRNA</td>
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<td>HeLa Plk4 shRNA</td>
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<td>MDA-MB-435 Plk4 shRNA</td>
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<tr>
<td>Plk4 +/- MEFs</td>
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<td>U2OS T-REx YFP-Plk4</td>
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<td>(0.01 µg/mL)</td>
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<td>U2OS T-REx YFP-Plk4</td>
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<td>(1 µg/mL)</td>
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<tr>
<td>HeLa T-REx Flag-Plk4</td>
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<td>HeLa T-REx Flag-Plk4</td>
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<td>(clone C1 0.1 µg/mL)</td>
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Table 5.1. Summary of effect of Plk4 manipulation on change in centriole number, and on migration in wound healing assays.

* vs. control:
MDA-MB-231 Plk4 shRNA: RFP shRNA
HeLa Plk4 siRNA: Luciferase siRNA
HeLa Plk4 shRNA: Luciferase shRNA
MDA-MB-435 Plk4 shRNA: Luciferase shRNA
Plk4 +/- MEFs: Plk4 +/- MEFs
HeLa T-REx Flag-Plk4: no tetracycline
U2OS T-REx YFP-Plk4: no tetracycline
5.3.2. Plk4-induced migration is resistant to suppression of Rac1 and Cdc42 activities

Plk4 has also been shown to activate Rac1, both by our group and others (Brashavitskaya, Kazazian et al. 2013, Godinho, Picone et al. 2014), and increased Rac1 activation at the leading edge stimulates lamellipodia formation and migration/invasion. We therefore explored the role of Rac1 in mediating the enhancement of cancer cell migration by Plk4. As expected, transfection of U2OS cells with dominant negative (DN) Rac1T17N at a transfection efficiency \( \approx 75\% \) slowed migration (Figure 5.2a,b). However, stimulation of U2OS migration by tetracycline-induced Plk4 expression was not significantly affected by the presence of DN Rac1 (Figure 5.2a). To confirm reduced GTP-bound Rac1 with transfection of YFP-Rac1T17N, I performed pulldown assays with PAK-PBD beads, and while I was able to show reduced endogenous active Rac1 with transfection of this construct, the YFP-Rac1T17N construct was also unexpectedly pulled down by the PAK-PBD beads in this assay (Figure 5.2c).
Figure 5.2

(a) Images showing the effect of YFP and YFP-DNRac1 expression on wound healing over time.

(b) Images demonstrating YFP and YFP-DNRac1 expression with Hoechst and Phalloidin staining.

(c) Gel image showing Rac1-GTP binding with YFP and YFP-DNRac1 expression, with PAK-PBD pulldown and nonspecific band analysis.

Gel Analysis:
- YFP: % control
- YFP-DNRac1: % control

Time (h) vs. Percent Wound Area Healed (%)
- YFP-DNRac1/tet +
- YFP-DNRac1/tet -
- YFP/tet +
- YFP/tet -

Rac1-GTP bound (% control)
- YFP
- YFP-DNRac1

Tet-

Tet+ YFP-Plk4

Pulldown:
- PAK-PBD
- Rac1-GTP
- YFP-DNRac1
- Nonspecific band

Total Lysate

kDa: 35, 25, 55

YFP: +, -
YFP-DNRac1: +, -
Figure 5.2. Plk4-induced activation of Rac1 does not solely mediate its effect on directional migration.  

a) Left panel, scratch-wound assays performed on U2OS T-REx YFP-Plk4 stable cells. Right panel, quantification demonstrates impairment of wound healing by YFP-DNRac1T17N, and enhanced directional migration with tetracycline-induced YFP-Plk4, which is not affected by YFP-DNRac1 expression (data points = percent of original wound area healed), n=4, *p<0.05 vs. no tetracycline/YFP-transfected cells.  

b) Representative immunofluorescence images of U2OS T-Rex YFP-Plk4 cells treated with (+) and without (-) tetracycline (0.1µg/mL X24h), and subsequently transfected with YFP or YFP-DNRac1T17N X24h (phalloidin, red; Hoechst, blue), showing efficient transfection in a wounding assay. Bar: 50µm.  
c) Representative immunoblot from a pull-down experiment to detect GTP-bound Rac1. Right panel, quantification of 3 experiments.
Directional migration towards a scratch wound is normally associated with the development of a polarized cell morphology, with the Golgi positioned on the side of the nucleus closest to the wound, as observed in Luciferase siRNA treated HeLa cells (Figure 5.3a). This re-orientation of the Golgi apparatus was impaired in HeLa cells that had been depleted of Plk4 with siRNA, reminiscent of the impaired Golgi reorientation in Plk4<sup>−/−</sup> MEFs shown in Chapter 3. Polarity and reorientation of the MTOC/Golgi apparatus in directional migration is a Cdc42 phenotype (Nobes and Hall 1999), and indeed Plk4 knockdown resulted in reduced levels of GTP-Cdc42 in HeLa cells while YFP-Plk4 expression induced in U2OS cells on tetracycline induction increased GTP-Cdc42 (Figure 5.3b). However, transfection of U2OS cells with DN Flag-Cdc42T17N at a transfection efficiency of ≈65-70% had no effect on migration speed in our assays (Figure 5.3c,e). In addition, transfection of HeLa cells with either the DN Flag-Cdc42T17N or the constitutively active (CA) Flag-Cdc42Q61L did not affect cell area in a spreading assay (Figure 5.3d). To confirm activity of the DN Flag-Cdc42T17N construct in cells, I assayed the development of front-to-rear polarity in HeLa cells using a scratch wound as for Plk4 siRNA in Figure 5.3a, immunostaining for α-tubulin as a marker for the MTOC. Re-orientation of the MTOC was impaired in HeLa cells that had been transfected with DN Flag-Cdc42T17N (Figure 5.3f), similar to the phenotype observed with Plk4 knockdown. Reduced endogenous GTP-Cdc42 was observed in U2OS cells in pulldown assays with PAK-PBD upon transfection with DN Flag-Cdc42T17N (Figure 5.3g), although again unexpectedly the Flag-Cdc42T17N was also pulled down in this assay. In summary, while Plk4 promotes Rac1 and Cdc42 activation, whether directly or indirectly, its promotion of migration might not be entirely attributable to signaling downstream of these Rho GTPases.
Figure 5.3

(a) Luciferase siRNA
GM130
Hoechst
Plk4 siRNA-B
Plk4 siRNA-C

Cells with re-oriented Golgi (%)

Luciferase siRNA
Plk4 siRNA-B
Plk4 siRNA-C

*  
**

(b) HeLa
Luciferase shRNA

Cdc42-GTP
Total Lysate

Cdc42-GTP
Total Cdc42

Luciferase shRNA
Plk4 shRNA#2
Plk4 shRNA#1

Cdc42-GTP bound (% control)

Cdc42-GTP
Total Lysate

Cdc42-GTP bound (% control)

Cdc42-GTP
Total Lysate

U2OS T-REX YFP-Plk4

Pulldown:
PAK-PBD

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Figure 5.3. Plk4 modulates Cdc42 activity and the development of cell polarity. a) Localization of Golgi (white arrows) identified by staining for GM130 (green), relative to the nucleus (Hoechst, blue), at 2h after wounding a monolayer of HeLa cells (orientation of wound indicated by white squares). Right panel shows percent of cells with appropriately localized (i.e. oriented towards the wound) Golgi, n=3, ≥30 cells scored in each by 2 independent blinded raters, *p<0.05, **p<0.005 vs. Luciferase siRNA. b) Representative immunoblots from pull-down experiments to detect GTP-bound Cdc42; short and long exposures shown. Bottom panels, quantification of 3-4 experiments. c) Left panel, scratch-wound assays performed on U2OS cells transfected with Flag or DN Flag-Cdc42T17N. Right panel, quantification of wound healing, n=3, p=0.98. d) Quantification of cell area of spreading HeLa cells transfected with Flag, CA Flag-Cdc42Q61L, or DN Flag-Cdc42T17N, n=2 experiments, 600 cells measured in each, p=0.91. e) Representative immunofluorescence images of U2OS transfected with Flag (green, top panels) or DN Flag-Cdc42T17N (green, bottom panels) X24h (phalloidin, red; Hoechst, blue), showing efficient transfection in a wounding assay. f) Localization of the MTOC (white arrows) identified by staining for α-tubulin (green), relative to the nucleus (Hoechst, blue), at 3h after wounding a monolayer of HeLa cells transfected with Flag (red, top panels) or DN Flag-Cdc42T17N (red, bottom panels) (orientation of wound identified using phalloidin immunostaining (far red, left panels) and indicated by white squares). Right panel shows percent of cells with appropriately localized (i.e. oriented towards the wound) MTOC, n=2, ≥20 cells scored in each, *p=0.003 vs. Flag. g) Representative immunoblot from a pull-down experiment to detect GTP-bound Cdc42. Right panel, quantification of 3 experiments. Bars: 50µm.
Another possibility was that Plk4 exerts a more direct effect on actin polymerization at the cell cortex, in keeping with the marked difference in actin cytoskeletal staining at the advancing front of migrating HeLa cells that had been depleted of Plk4, as compared to control (Figure 5.4a). This is reminiscent of the difference in actin staining observed between migrating Plk4 heterozygous and wildtype MEFs (Rosario, Kazazian et al. 2014).

5.3.3. Plk4 interacts physically and functionally with the Arp2/3 complex member Arp2

In an unbiased screen searching for motility-related candidate Plk4 interacting proteins by proximity-dependent biotin identification (BioID) mass spectrometry in HEK293 cells, we identified several members of the Arp2/3 complex (Figure 5.4b,c Table 5.2), the key regulator of branched actin network formation. The Mass Spec analysis was performed by Christopher Go in the laboratory of Anne-Claude Gingras. Criteria for significance included an iProphet protein probability \( \geq 0.95 \) and a minimum of 2 unique peptides, corresponding to an estimated protein level false-discovery rate (BFDR) of \( \leq 0.02 \). I therefore interrogated a panel of Arp2/3 complex proteins in reciprocal co-immunoprecipitation experiments with Plk4 (Figure 5.4d). Plk4 interacted specifically with Arp2 (Figure 5.4d,e), an interaction for which the Plk4 polobox 1 and 2 (PB1-2) domain was required and sufficient (Figure 5.5a). This interaction was in keeping with an observed effect of Plk4 on actin polymerization at the cell cortex, as suggested by the marked difference in actin cytoskeletal staining at the advancing front of migrating HeLa cells that had been depleted of Plk4, as compared to control cells (Figure 5.4a). Arp2 contains a consensus sequence for phosphorylation by Plk4, centered at T237/T238, which has recently been identified as an activation site (Figure 5.5b; (LeClaire, Baumgartner et al. 2008, Narayanan, LeClaire et al. 2011)). I generated an Arp2 T237/238A mutant, which showed reduced interaction with Flag-Plk4 in co-immunoprecipitation assays (Figure 5.5c).
Figure 5.4

a) Immunofluorescence images of cells expressing Flag-Plk4 and RFP-Arp2, with Hoechst staining for nuclei.

b) Pie chart showing the distribution of proteins in different cellular structures:
- Centrosome
- Actin cytoskeleton
- Other

Proteins: ALMS1, CENPJ, CEP192, CEP152, CEP350, CEP63, CEP85, FGFR1OP, GNAI2, NEDD1, NME7, SASS6, STIL, ACTA1, ACTBL2, ACTR2/ARP2, ACTR3/ARP3, AIF1L, ARPC1A, ARPC2, ARPC5, ARPC5L, CAPZA2, CDC42BPA, FLII, GAS2L3, GSN, MPRIP, NEXN, TMOD2, TWF1, WDR1.

c) Western blot analysis of BirA*-Flag, Flag, BirA*-Flag-Plk4, and BirA*-Flag-Plk4 with different treatments.

Tet (µg/mL) = 2, 0, 0.2, 2

kDa = 130, 25, 55

β-tubulin

d) Western blot analysis of Flag-Plk4 and RFP-Arps with different treatments.

Flag-Plk4, Flag, RFP-Arp2, RFP-Arp3, RFP-ArpC1, RFP-ArpC3, RFP-ArpC5, RFP-ArpC5L

Input: RFP, IB: Flag

Input: RFP, IB: RFP

Input: Flag, IB: RFP

Input: Flag, IB: Flag

Input: RFP

Input: Flag

Flag-Plk4

Flag

RFP-Arp2

RFP-Arp3

RFP-ArpC1

RFP-ArpC3

RFP-ArpC5

RFP-ArpC5L

IgG

IgG

E) Western blot analysis of Flag-Plk4 and RFP-Arps with different treatments.

Input: RFP, IB: Flag

Input: RFP, IB: RFP

Input: Flag, IB: RFP

Input: Flag, IB: Flag

Input: RFP

Input: Flag

Flag-Plk4

RFP-Arp2

RFP-Arp3

RFP-ArpC1

RFP-ArpC3

RFP-ArpC5

RFP-ArpC5L

IgG

IgG

156
**Figure 5.4. Plk4 interacts with the Arp2/3 complex.** a) Representative images showing reorganization of actin filaments (red) after scratch wound, markedly suppressed in Plk4 siRNA-treated cells (orientation of wound indicated by white squares). Bars: 50µm, inset 20µm.

b) Diagram of high confidence protein interactions for Plk4, discovered by BioID. Purple indicates centrosomal proteins, green proteins involved in regulation of the actin cytoskeleton, and blue others. Based on n=2 biological replicates.

c) Immunoblot confirming expression of BirA*-Flag or BirA*-Flag-Plk4 in HEK293 T-REx cell lines upon tetracycline induction, using anti-Flag antibody with β-tubulin as a loading control.

d) Immunoblots showing reciprocal coimmunoprecipitation of Flag-Plk4 and RFP-Arp2/3 complex subunits, as indicated, after coexpression in HEK293T cells.

e) Immunoblots from reciprocal coimmunoprecipitation of Flag-Plk4 and RFP-Arp2 after coexpression in HEK293T cells, using anti-Flag and anti-RFP antibodies.
Table 5.2. List of high confidence preys of PLK4, post SAINTexpress (v 3.3) analysis after filtering by BFDR less than or equal to 0.02. Prey Gene is as per NCBI Entrez Gene, Prey Accession is the NCBI protein accession number; Average Spectra is the average of spectra identified for the prey in both replicates; Average Control Spectra is the average spectra found across the 8 controls for a prey; AvgP (average probability across replicates); Fold Change (sum total of counts in the purification divided by counts in the controls; If necessary, fold change column will have a value of 0.1 added to cell to prevent divisions by 0.); and Bayesian FDR are listed for each bait-prey relationship. Note that PLK4 wildtype and PLK4 S286A/T290A replicates were merged for SAINT analysis. * in Actin and Centrosome Functional Annotation columns indicates previously reported interactors based on BioGRID (PMID: 26728913) and InAct (PMID: 2212220); annotations were selected from GO Biological Process terms and, if necessary, GO Molecular Function terms and left empty if no annotations were present in UniProt.

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**Figure 5.5**

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**b**

Plk4 phosphorylation motif:

- $\delta$ TT γ X γ X $\delta$

Arp2 (236-242) WT:

- $E$ TT VL V E-

Arp2 (236-242) T237/238A:

- $E$ AA VL V E-

$\delta$ = charged

γ = large hydrophobic

X = any

### c

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$\delta$ = charged

γ = large hydrophobic

X = any

### Plk4

**Kinase domain**

- FL
- 1-374
- 624-902
- 878-970

**Arp 2**

- +
- -
- +
- -
**Figure 5.5. RFP-Arp2 interacts with the Plk4 PB1-2 domain.**  
a) Immunoblots showing reciprocal coimmunoprecipitation of Flag-Plk4 fragments coexpressed with RFP-Arp2 in HEK293T cells. Bottom panel, scheme of expressed Plk4 fragments coexpressed with RFP-Arp2 in HEK293T cells, where (+) indicates interaction.  
b) Predicted Plk4 phosphorylation motif in Arp2 from residue E236 to E242, with potential phosphorylation sites marked in red and T to A mutations marked in blue.  
c) Immunoblots showing reciprocal coimmunoprecipitation of Flag-Plk4 coexpressed with RFP-Arp2 WT or RFP-Arp2 T237/238A in HEK293T cells.
In HeLa cells migrating towards a scratch wound, RFP-Arp2 and Flag-Plk4 localized similarly to the lamellopodial front, as did endogenous Arp2 and Flag-Plk4 (Figure 5.6a). It is possible that in wound healing assays any cytoplasmic protein may localize to the ruffling lamellipodia, which often contains cytoplasm. By contrast to Plk4 and Arp2, the unrelated Rho GEF PLEKHG6, which contains the Plk4 consensus phosphorylation motif, and distributes widely in the cytoplasm, did not concentrate with Plk4 at the leading edge (Figure 5.6b).
Figure 5.6

(a) Wounding assay

(b) Wounding assay
Figure 5.6. Plk4 localizes with Arp2 to the protrusions of motile cells. a) Representative immunofluorescence images of HeLa cells transfected with Flag-Plk4 +/- RFP-Arp2. Confluent cells were wounded then fixed and labelled using anti-Flag antibodies (green), phalloidin (far red, left panel), Hoechst (blue) and RFP (visualized directly, red, top panels) or Arp2 antibody (red, bottom panels). Merged images demonstrate regions of overlap (yellow) between Flag-Plk4 and RFP-Arp2 (top panels) or endogenous Arp2 (bottom panels) at the lamellipodia (orientation of wound indicated by white squares). b) Representative immunofluorescence images of HeLa cells transfected with Flag-Plk4 and RFP-Arp2 (top and middle panels) or with Flag-Plk4 and YFP-PLEKHG6 (bottom panels). Confluent cells were wounded then fixed and labelled using anti-Flag antibodies (green, top and middle panels or red, bottom panels), phalloidin (far red, left panels) and Hoechst (blue). Merged images demonstrate enrichment at the lamellipodia with Flag-Plk4 and RFP-Arp2, while YFP-PLEKHG6 stains diffusely in the cytoplasm (orientation of wound indicated by white squares). Bars: 20µm.
Using a phospho-specific anti-Arp2 T237+T238 antibody, we found that Arp2 phosphorylation at T237+T238 was significantly greater in HeLa cells transfected with wildtype Flag-Plk4, than cells transfected with kinase-dead Flag-Plk4 K41M (Figure 5.7a). Furthermore, cells depleted of Plk4 by means of shRNA had lower levels of T237+T238 phosphorylated Arp2 than control Luciferase shRNA (Figure 5.7a). These data were in keeping with the possible phosphorylation of Arp2 by Plk4 at this site. However, cells were pre-treated with pervanadate, a potent tyrosine phosphatase inhibitor, in the absence of which the predicted phospho-Arp2 T237+T238 band was not present on the immunoblot (Figure 5.7b). Since widespread tyrosine phosphorylation could induce a cascade of secondary molecular events whereby Arp2 could be non-specifically phosphorylated at T237+T238, it was challenging to conclude that physiologically relevant phosphorylation of Arp2 by Plk4 was occurring at this site. An in vitro kinase assay was therefore performed, and showed dose-dependent phosphorylation of wildtype Arp2 in the presence of wildtype Plk4 (Figure 5.7c, top panels), while the T237/238A mutant Arp2 was not phosphorylated, suggesting that this is indeed a bona fide Plk4 phosphorylation site (Figure 5.7c, bottom panels).
**Figure 5.7**

**a**

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**Fold change of phospho-Arp2 (of wild type)**

- Green bar: Flag-Plk4
- Green bar with asterisk: Flag-Plk4 K41M

**b**

- Pervanadate: + - - + + +
- Flag-Plk4: - + + + - +
- Flag-Plk4 K41M: - - - - + -
- Flag-Arp2: + + + + + -
- Flag: - - - - - +
- λPPase: - - + - - -

**Fold change of phospho-Arp2 (Luciferase=1)**

- Luciferase shRNA: + - - - -
- Plk4 shRNA#1: - + + - -
- Plk4 shRNA#2: - - - + +

**c**

- Flag-Plk4 WT: + - - + + +
- Flag-Plk4 K41M: - + + - - -
- Arp2: + + +
- Arp2 T237/238A: - + +

**IB: phospho-Arp2**

- Flag-Arp2
- Arp2
- Flag
- Flag-Arp2
- Tubulin

**IB: Arp2**

- 55 kDa

**IB: Flag**

- 130, 100, 55 kDa

**IB: Tubulin**

- 50 kDa

**Autorad and Colloidal Blue**

- Flag-Arp2
- Flag-Plk4
- Flag-Arp2

- Flag-Plk4
- Flag-Arp2
**Figure 5.7. Plk4 phosphorylates Arp2.** a) Immunoblots from HeLa cell lysates transfected with Flag-Plk4-WT or Flag-Plk4-K41M (kinase-dead) and Flag-Arp2 (left panels), or from Luciferase and Plk4 shRNA stable cells transfected with Flag-Arp2 (right panels), showing reduced phosphorylated-Arp2 with Flag-Plk4-K41M vs. WT (left panels) and with Plk4 vs. Luciferase shRNA (right panels), using a phospho-specific anti-Arp2 (phospho T237+T238) antibody. Quantification of fold change in phospho-Arp2 relative to total Arp2, *p=0.007 vs. WT, n=4 and **p<0.05 vs. Luciferase shRNA, n=3. b) Immunoblots from HeLa cell lysates transiently transfected with Flag-Plk4 WT or kinase-dead Flag-Plk4 K41M and Flag-Arp2 or Flag, probed with the indicated antibodies, testing specificity of the phospho-specific anti-Arp2 T237+T238 antibody. ∆ indicates non-specific band. c) Purified Flag-Plk4 WT but not K41M phosphorylates Arp2 in an *in vitro* kinase assay. Less phosphorylation of Arp2-T237/238A mutant was observed (bottom panel).
As predicted based on the key role of the Arp2/3 complex in terminal signaling for actin polymerization, depletion of Arp2 by siRNA suppressed spreading of HeLa cells (pool of 4, and 3 of 4 individual constructs) (Figure 5.8a,b). The small molecule Arp2/3 complex inhibitor CK-666, which blocks activation by preventing conformational change of the complex, also impaired HeLa spreading (Figure 5.8c,d). Inactivation or depletion of Arp2 also impaired directional migration (Figure 5.8e,f), similar to the impaired migration seen with Plk4 knockdown or inhibition.
Figure 5.8

(a) Relative Arp2 mRNA expression (ΔCt)

(b) Luciferase siRNA

(c) DMSO

(d) Cell Area (µm²)

(e) 0h 4h 8h 12h

(f) Percent Wound Area Healed (%)
Figure 5.8. Arp2 promotes cancer cell spreading and migration. a) Relative Arp2 mRNA levels in HeLa cells transfected with Arp2 vs. Luciferase siRNAs X48h. GAPDH used for internal control. b) Representative immunofluorescence images of spreading HeLa cells transfected with Arp2 siRNAs or Luciferase siRNA X48h (phalloidin, green; Hoechst, blue). Quantification of cell area (right panel), showing reduced spreading with 3 of 4 Arp2 siRNAs, n=3 experiments, 500 cells measured in each, *p<0.0001 vs. Luciferase siRNA. c) Representative immunofluorescence images of spreading HeLa cells treated with 50 µM of Arp2/3 inhibitor (CK-666) for 16h (phalloidin, green; Hoechst, blue). Quantification of cell area (right panel) showing dependence of spreading on Arp2/3 complex activity, n=6 experiments, 500 cells measured in each, *p=0.02 vs. DMSO. d) Representative immunofluorescence images of spreading HeLa cells pretreated with indicated doses of CK-666 X16h (phalloidin, green; Hoechst, blue). Right panel, quantification of cell area, n=6 experiments, 500 cells measured in each, *p<0.0001 vs. DMSO. Cell area quantified using INCell Analyzer 6000. Bars: 50 µm. e) Top panel, scratch-wound assay performed on confluent HeLa cells treated with 50µM CK-666 X6h. Bottom panel, quantification demonstrates impaired directional migration with CK-666 (data points = percent of original wound area healed), n=3, *p<0.01, **p<0.001 vs. DMSO. f) Top panels, scratch-wound assays performed on HeLa cells transfected with Arp2 vs. Luciferase siRNAs X48h. Bottom panel, quantification demonstrates impaired directional migration in cells with Arp2 knockdown (data points = percent of original wound area healed at the indicated time), n=2, *p<0.01 vs. Arp2 siRNA-pool, -01, -02, -03, -04.
To investigate whether the stimulatory effect of Plk4 on motility could be mediated through Arp2, I evaluated spreading by HeLa cells depleted of Arp2 with siRNA and transfected with Flag-Plk4. The enhancement of spreading by Flag-Plk4 observed in Luciferase siRNA control cells was absent in Arp2-depleted cells (Figure 5.9a). In addition, the inhibitory effect of Arp2 siRNA on spreading could be partially rescued by adding back WT but not T237/238A mutant Arp2, in keeping with promotion of cell spreading by Plk4 through phosphorylation of Arp2 at this site (Figure 5.9a). Depletion of Arp2 had no effect on centriole number, either in otherwise untreated cells or with Plk4-induced centriole amplification (Figure 5.9b), demonstrating separation of the two Plk4 functions. Furthermore, the enhanced wound healing seen in U2OS cells stably expressing YFP-Plk4 on tetracycline induction was diminished by the inhibitor CK-666 (Figure 5.9c). Arp2 did not localize to the leading edge in Plk4-depleted HeLa cells engaged in wound healing at 3h, coincident with impaired lamellipodia formation in these cells (Figure 5.9d). Taken together, these results indicate that Arp2 binds to Plk4, is phosphorylated and activated in its presence, and that this activation is important for the stimulation of cancer cell motility by Plk4.
Figure 5.9

(a) Bar graphs showing cell area measurements for various treatments. The x-axis represents different treatments, and the y-axis shows cell area in square micrometers. Each bar is labeled with the treatment designation.

(b) Microscopy images of U2OS T-REx YFP-Plk4 under different conditions. The images show centrin and YFP-Plk4 fluorescence, with Hoechst dye for nuclei staining. The images are labeled with treatment conditions: Luc, Arp2 siRNA-03, and Arp2 siRNA-04.

(c) Percentage of cells with centrin-positive foci under different treatments. The x-axis represents treatment conditions, and the y-axis shows percentage of centrin-positive cells. The bars are color-coded to indicate different focal counts.

(d) Percent wound area healed over time for different treatments. The x-axis represents time in hours, and the y-axis shows percentage of wound area healed. The graph includes data points for DMSO/tet, CK-666/tet, and CK-666/tet+.
Figure 5.9. Plk4 interacts functionally with Arp2 to facilitate spreading and migration.

a) Left panel, quantification of cell area of spreading HeLa cells transfected with Arp2 or Luciferase siRNA X48h, then transfected with Flag or Flag-Plk4 X18h, showing suppression of increased area on Flag-Plk4 transfection when cells are treated with Arp2 siRNAs, n=4, *p=0.04. Right panel, quantification of cell area of spreading HeLa cells transfected with Arp2 or Luciferase siRNAs X36h, then transfected with siRNA-resistant WT Arp2 (RFP-Arp2-WTΔ) or siRNA-resistant T237/238A Arp2 (RFP-Arp2-T237/238AΔ) showing failure of T237/238A Arp2 to rescue spreading upon Arp2 depletion, n=3, *p<0.01 vs. siArp2-03 with RFP-Arp2-WTΔ. b) Representative immunofluorescence images of U2OS T-REx YFP-Plk4 cells transfected with Luciferase or Arp2 siRNAs X48h, and with Plk4 expression induced by 0.1µg/mL tetracycline. Cells were labeled with anti-centrin (red) and Hoechst (blue). The right panels/inserts show magnified centrosomes (boxed in white) for each condition. Bottom panel, bar graph showing frequency of cells with indicated number of centrioles per cell, which is not affected by Arp2 knockdown. Bars: 10µm, inset 1µm. c) Top panels, scratch-wound assay performed on U2OS T-REx YFP-Plk4 cells with (+) or without (-) tetracycline, and treated with CK-666 or DMSO. Bottom panel, failure of Plk4 to stimulate wound healing after CK-666 treatment, n=3, *p<0.01 vs. DMSO/tet-. d) Representative immunofluorescence images of HeLa Luciferase or Plk4 shRNA cells transfected with RFP-Arp2 (red). Confluent cells were wounded, then fixed and labelled using phalloidin (green) and Hoechst (blue). There is a lack of protrusional RFP-Arp2 in Plk4 shRNA HeLa cells at 3h (orientation of wound indicated by white squares). Bar: 20 µm.
5.4 Discussion

The presence of supernumerary centrosomes has been documented in almost all varieties of human cancer (Chan 2011), and evidence has been generated to imply a causative role of centrosomal amplification (CA) in cancer development and progression (Pihan, Purohit et al. 2001, Ganem, Godinho et al. 2009, Reiter, Gais et al. 2009). One proposed mechanism is through spindle anomalies, and resultant errors in chromosome division. Godinho and colleagues have proposed another potential mechanism through which supernumerary centrioles may facilitate carcinogenesis, namely through enhanced cell migration and invasion, where CA disrupts normal cell-cell adhesion because of Rac1 activation through effects on centrosomal microtubule nucleation. In their model, centriole number was manipulated by altering Plk4 levels or through cytokinesis failure (Godinho, Picone et al. 2014). While Pellman’s group proposes that CA promotes invasion independent of Plk4, when we altered Plk4 levels we did not find that the degree of CA consistently correlated with the degree of change in migration or invasion in our assays, as compared to controls. In fact, Plk4+/− MEFs, which exhibit CA, show reduced migration and invasion compared to wildtype (Figure 3.3a, (Rosario, Ko et al. 2010, Rosario, Kazazian et al. 2015)).

Godinho and colleagues used transiently overexpressed truncated Plk41–608, which retains kinase activity but does not induce CA (Guderian, Westendorf et al. 2010), as a negative control to show that Plk4 overexpression without CA does not result in the formation of invasive protrusions. While Plk41–608 (kinase domain) is active as a kinase, it does not dimerize owing to truncation of its C-terminal PB domains and therefore does not display activity in effecting centriole amplification (Guderian, Westendorf et al. 2010). Given the importance of the Plk4 PB1-2 and PB3 domains in Plk4 localization, homodimer formation and interactor binding
(Dzhindzhev, Yu et al. 2010, Slevin, Nye et al. 2012, Klebba, Buster et al. 2015), it is likely that important aspects of Plk4’s functionality are missing in this system. Furthermore, in their cytokinesis failure CA system, it could be suggested that since there is likely Plk4 expressed at the multiple centrioles in cells with CA, Plk4 is in fact overexpressed in these cells compared to the “evolved” cells without CA. Recently, other investigators have also described the acquisition of an invasive phenotype in noncancerous cells following manipulations that increased Plk4 expression and cellular centriole number, as in the study by Godinho et al. (Denu, Zasadil et al. 2016). However, further investigation would be required to support the hypothesis that centrosome amplification independent of Plk4 is the cause of increased invasiveness.

The family of small Rho GTPases, in particular the three most well known members RhoA, Rac1 and Cdc42, together regulate cytoskeletal dynamics. Live imaging using biosensors has shown that their activated forms localize to the leading edge of migrating cells, in a pattern that is similar to what we observed for Plk4. Plk4 has previously been shown to increase Rac1 activity (Brashavitskaya, Kazazian et al. 2013, Godinho, Picone et al. 2014). As noted above, Godinho and colleagues suggested that this is triggered by CA and mediates increased cellular invasiveness. However, the Plk4-mediated increase in GTP-bound Rac1 (Godinho, Picone et al. 2014) could not fully explain the Plk4-induced enhancement of motility in our wound healing assays. For example, in U2OS cells expressing Plk4 on tetracycline induction, transfection with dominant negative Rac1T17N, which alone reduces cell migration as expected due to its critical role in lamellipodia formation, did not abrogate the Plk4-induced promotion of cell migration. Here overexpression of the dominant negative (T17N) mutant versions of Rac1 or Cdc42, which due to their high-affinity state toward upstream GEFs create a situation in which these mutants
sequester their respective GEFs, decreases activation of the corresponding endogenous Rho GTPase (Rossman, Der et al. 2005).

Consistent with the impaired positioning of the Golgi/MTOC in Plk4+/− MEFs compared to wildtype in a directional migration assay, there was impaired reorientation of the Golgi in front of the nucleus in migrating HeLa cells depleted of Plk4. As this is a Cdc42 phenotype, I did explore the effect of Plk4 on Cdc42 activation. While Plk4 modulated levels of active Cdc42, DN Cdc42T17N had no effect on directional migration in U2OS cells or spreading in HeLa cells. This is may be a reflection of cooperation amongst the RhoGTPases in facilitating cell motility. Alternatively, the contribution of individual RhoGTPases in the regulation of cell migration can be cell type dependent, which is likely to be dictated by the relative abundance of related GTPases and the availability of specific downstream effectors. For example, while Rac1 is an important regulator of migration speed in fibroblasts (Vidali, Chen et al. 2006) Rac1 deficient macrophages, which also express Rac2, do not exhibit defects in migration (Wells, Walmsley et al. 2004). In addition, while the loss of Cdc42 function in fibroblasts impairs migration speed (Yang, Wang et al. 2006), there is no effect on migration of fibroblastoid cells (Czuchra, Wu et al. 2005), and macrophage migration is enhanced (Allen, Zicha et al. 1998). Thus, while Plk4 regulates Rac1 and Cdc42 activation, whether directly or indirectly, the results presented in this chapter suggest that Plk4’s promotion of migration in the cell types assayed is not solely attributable to signaling downstream of these RhoGTPases.

Affinity-based activation assays to detect GTP-bound Rac1/Cdc42 are widely used for the purpose of assessing Rho GTPase activity in biological studies. These assays use the binding domains in Rho GTPase effector proteins that specifically recognize the active, GTP-bound form of the Rho GTPase to measure activation. The p21-activated kinase 1 (Pak1) contains a
regulatory site from amino acids 74 to 89 for interaction with the active GTP-bound forms of Rac1/Cdc42 called the CRIB domain (Cdc42/Rac interactive binding domain) or p21-binding domain (PBD). Using such a probe, it is possible to selectively bind GTP-bound, active Rac1/Cdc42. The isolated GTP–bound GTPase is then detected through the use of specific anti-Rac1 or anti-Cdc42 antibodies. As the Pak1-PBD domain is specific for GTP-bound Rac1/Cdc42, I used this assay to confirm reduced active Rac1/Cdc42 following transfection with the respective dominant negative constructs. DNRac1T17N and DNCdc42T17N contain an asparagine substitution that abolishes the affinity for GTP and reduces affinity for GDP, thus binding strongly to GEFs and competing with wild type Cdc42/Rac1 for GEF binding and preventing their activation. Curiously, when we performed these assays, the YFP-DNRac1T17N and Flag-DNCdc42T17N proteins were also pulled down by Pak1-PBD. Therefore, while there certainly appeared to be less endogenous wild type Cdc42 or Rac1 following transfection with the dominant negative constructs compared to control, this assay could not conclusively confirm this. An alternative interpretation was that there was altered competition for the Pak1-PBD beads. In performing a literature search and consulting a principal investigator with considerable experience with Rho GTPases and activation assays (Dr. K. Szaszi), we could not find evidence of similar activation assays being performed using the dominant negative Rac1 or Cdc42 constructs, although one paper did show that in a RhoA activation assay with Rhotekin-RBD, DNRhoAT19N was not detected by Western blot analysis of precipitated proteins with RhoA antibody (Stofega, DerMardirossian et al. 2006). To test whether our results were due to non-specific binding of the dominant negative constructs to the beads themselves, I incubated the same lysate from the DNRac1T17N transfected cells with Rhotekin-RBD beads specific for GTP-RhoA. Neither endogenous nor DNRac1T17N bound the Rhotekin-RBD beads in Western
blot analysis using an anti-Rac1 antibody. Further optimization of this assay is required. I did confirm that the dominant negative constructs resulted in functional inhibition in cells as expected, where DNARac1T17N transfection in U2OS cells delayed wound healing migration and DNCdc42T17N transfection resulted in impaired re-orientation of the MTOC in a wound healing assay. While I used the same conditions as for the wound healing assays to prepare the cell lysates for the activation assay, it is likely that as a result I was loading such a large amount of YFP-DNARac1T17N and Flag-DNCdc42T17N on the Pak1-PBD beads that these were being precipitated in addition to the active, endogenous forms. The amount of lysate could be titrated, as well as the amount of beads used, although this was optimized for detection of the wild type Cdc42/Rac1. The activation assay may also be made specific for Cdc42 alone by using the WASP Cdc42 binding region. This may also increase the sensitivity of the assay for endogenous GTP-Cdc42, which was of low abundance and difficult to detect in U2OS cells, as the more abundant active Rac1 would not compete for WASP Cdc42 binding domain.

To search for other potential interactors that could mediate Plk4’s effect on cell motility, we performed an unbiased screen searching for candidate Plk4 interacting proteins by mass spectrometry. Initial attempts with Flag affinity purification followed by MS/MS were not successful in identifying high confidence Plk4-interacting proteins, other than CEP152, likely due to Plk4’s inherent instability and relative paucity of protein expression. Several optimizations were attempted, including using both HeLa and HEK293 Flag-Plk4 inducible cell lines, use of 10 15-cm plates per biological replicate and sonication, to no great effect. During the course of my studies, BioID was established as an effective method to screen for weak or transient protein interactions. In BioID, a promiscuous biotin ligase BirA is fused to the protein of interest and expressed in cells. In the presence of biotin, it biotinylates proximal endogenous
proteins and enables their selective isolation and identification with streptavidin affinity purification followed by mass spectrometry. BioID identified several members of the Arp2/3 complex as potential mediators of Plk4-induced motility. Here, supporting a more direct role for Plk4 in regulating actin polymerization, we confirm a novel physical interaction of Arp2 with Plk4 by co-immunoprecipitation, show a similar localization to lamellipodia in motile cells, a similar effect on motility, and phosphorylation of Arp2 by Plk4 implicating the Arp2/3 complex as an effector downstream of Plk4 to facilitate cancer cell motility and invasion.

As expected due to its role in the dynamic remodeling of the actin cytoskeleton, aberrant Arp2/3 complex function has been implicated in a number of disease conditions, and an abnormal expression level of Arp2/3 complex members has been shown in several types of cancer. Interestingly, in gastric cancer the expression level of Arp2/3 appears to be contrasting at the mRNA and protein levels. Using quantitative real-time RT-PCR, a study by Kaneda et al. (Kaneda, Kaminishi et al. 2002) found reduced expression of the p41Arc subunit in gastric cancer tissues and cell lines. Another study by the same group found that all seven subunits of the Arp2/3 complex showed decreased expression levels in 32 primary gastric cancer samples (Kaneda, Kaminishi et al. 2004). Contrastingly, Zheng et al. (Zheng, Zheng et al. 2008) showed that both Arp2 and Arp3 are increased in gastric cancer tissues using immunohistochemistry, and Sun et al. (Sun, Shang et al. 2014) similarly found increased Arp2 in gastric cancer tissues, with high Arp2 expression proposed to be involved in gastric cancer invasion and metastasis/worse survival. Immunohistochemical analysis has also identified Arp2 as overexpressed in lung and colorectal cancers, and in lung cancer is suggested to be involved in aggressive tumour behaviours (Otsubo, Iwaya et al. 2004, Semba, Iwaya et al. 2006).

Arp2 is suggested to have a modulating role in cancer invasion, where increased cell motility is
one of the main characteristics of cancer cells with metastatic potential. As expected due to the role of the Arp2/3 complex in actin nucleation and actin filament branching, we show that Arp2 knockdown in HeLa cells impaired spreading and migration, similar to Plk4. The Arp2/3 complex is localized to lamellipodia, and several knockdown studies have demonstrated that in MEFs lamellipodial protrusion is inhibited when Arp2/3 activity is reduced, thus impairing directional cell migration (Bailly, Ichetovkin et al. 2001, Rogers, Wiedemann et al. 2003, Suraneni, Rubinstein et al. 2012, Wu, Asokan et al. 2012). In cancer cells, Rauhala et al. (Rauhala, Teppo et al. 2013) showed that silencing of the Arp2/3 complex subunits, including Arp2, typically decreased pancreatic cancer cell migration, and Sun et al. also showed in MKN28-M cells, a subline of MKN-28 gastric cancer cells with high metastatic potential, that Arp2 silencing using siRNA also delayed cell migration, leading to reduced scratch wound closure, and suppressed cell invasion and migration in Boyden chamber assays (Sun, Shang et al. 2014). While Sun and colleagues showed that ectopic expression of Arp2 promoted the migration and invasion of MKN28-NM cells (reduced metastatic potential) in wound healing and Boyden chamber assays (Sun, Shang et al. 2014), I did not see a significant increase in spreading or migration of HeLa cells on transient transfection with RFP-Arp2. This may be due to the already inherently high spreading/migration potential of HeLa cells, where additional Arp2 mediated actin polymerization would not exhibit a marked effect. Moreover, the Arp2/3 complex is regulated and activated by the members of WASP family, including WASP, N-WASP, and Scar/WAVE, as well as phosphorylation, and Arp2 expression may not be the rate limiting factor in cancer cells in Arp2/3 complex mediated cell spreading/migration. Finally, not all cell motility is dependent on Arp2/3 complex activity (Wu, Asokan et al. 2012). Wu et al. (Wu, Asokan et al. 2012) also showed that knockdown of Arp2 using shRNA in MEFs results in a
marked reduction in migration speed. A similar reduction of cell migration speed was observed when MEFs were treated with the Arp2/3 inhibitor CK-666 (100 mM), and depletion of Arp2/3 using shRNA’s targeting both p34Arc and Arp2 led to defects in cell spreading (Wu, Asokan et al. 2012). In our studies, inhibition of Arp2/3 complex activity using CK-666 similarly impaired cancer cell spreading and migration, and this phenomenon was consistent with previous studies; Interestingly, in MEFs, but not CK-666–treated A2780 cells, 2D migration was impaired, while A2780 cancer cells with a very low basal level of cell invasion had no change in migration speed in a wounding assay (Wu, Asokan et al. 2012, Paul, Allen et al. 2015). These data indicate that in cancer cells Arp2/3 is not always required for migration.

In our studies, Arp2 knockdown antagonized the increased cell spreading caused by Plk4 upregulation. Moreover, ectopic expression of Plk4 promoted cancer cell migration, and this effect was attenuated by Arp2/3 inhibition. Therefore, these results indicate that Plk4 function in cell motility depends on its co-existence with Arp2. Furthermore, these are the first studies to describe that Plk4 functions in cancer invasion and metastasis through coordination with the Arp2/3 complex. Therefore, determining Arp2 expression levels may be crucial in the rational selection of patients treated with Plk4 inhibitors. Ascertaining the effect of Plk4 inhibition on the invasion/metastasis of cancer cells with high vs. low Arp2 levels in preclinical models is an important next step.

Until recently the Arp2/3 complex was thought to be primarily regulated by WASP proteins; However, recent evidence indicates that it is also phosphorylated by serine-threonine kinases. LeClaire et al. (LeClaire, Baumgartner et al. 2008) have shown that phosphorylation on threonine and tyrosine residues of the Arp2 subunit is functionally necessary to promote the actin nucleating activity of the Arp2/3 complex. Using mass spectrometry and mutagenesis studies,
phosphorylation of Arp2 at T237, T238 and Y202 was identified, and was shown to be necessary for activation of the Arp2/3 complex in pyrene-labeled actin assays in the presence of NPFs. In addition, expression of Arp2 with alanine substitutions at T237/238 and Y202 in Drosophila melanogaster Schneider S2 cells did not restore lamellipodia in cells treated with Arp2 siRNA, indicating that phosphorylation of Arp2 is functionally relevant for lamellipodia formation (LeClaire, Baumgartner et al. 2008). Narayan et al. (Narayan, LeClaire et al. 2011) then used computational simulations to show that phosphorylation of Arp2 at T237 and T238, which are near the interface of Arp3, ARPC2, and ARPC4 at the core of the complex, results in conformational changes that are permissive for reorientation of Arp2 relative to Arp3, allowing the complex to change toward a short-pitch dimer orientation required for activity. They therefore proposed a model in which phosphorylation destabilizes the inactive state, allowing Arp2 to reorient itself into a state that is permissive for full activation by NPFs (Narayan, LeClaire et al. 2011). More recently, this group then went on to identify the first kinase shown to phosphorylate and increase the activity of the Arp2/3 complex, the Nck-interacting kinase (NIK), a Ste20/MAP4K4 serine/threonine kinase (LeClaire, Rana et al. 2015). NIK was shown to directly bind and phosphorylate Arp2, increasing actin nucleating activity of the Arp2/3 complex and promoting plasma membrane protrusion in response to epidermal growth factor (LeClaire, Rana et al. 2015). Here we show that Plk4 may phosphorylate Arp2 using both a phospho-Arp2 T237+T238 antibody and an in vitro kinase assay, and this appears to be specific to the T237 and T238 residues. In experiments utilizing the phospho-Arp2 T237+T238 antibody, cells were pre-treated with pervanadate, a potent tyrosine phosphatase inhibitor, in order to visualize a band of the appropriate molecular weight on immunoblots. While pervanadate would inhibit loss of tyrosine phosphorylation, Plk4 is a serine-threonine kinase and this antibody recognizes
phosphorylated threonine residues, indicating a potential issue with the specificity of this antibody. Nevertheless, this may be reflective of a requirement for Y202 phosphorylation to permit phosphorylation of the T237+T238; The activation sites of Arp2 are T237/238 and Y202, although neither a kinase that phosphorylates the tyrosine residue, nor the functional significance of Y202 phosphorylation are known. I attempted to pretreat cells with Calyculin A, a serine/threonine phosphatase inhibitor, but this was highly toxic to all the cell types tested at varying concentrations. An in vitro kinase assay was therefore used to assess phosphorylation of Arp2 by Plk4. Interestingly, in co-immunoprecipitation assays the Arp2 T237/238A mutant showed reduced interaction with Plk4 when compared to wildtype Arp2, suggesting that this site is important for interaction with Plk4, although interpretation of this result is difficult.

Phosphorylation of Arp2 by Plk4, as in the kinase assays described in this thesis, suggests that Plk4’s effect on cell motility and invasion may be mediated by the increased actin nucleating activity of the Arp2/3 complex. Plk4 has also been shown to increase Rac1 activity by our group, as well as by Godinho and colleagues (Godinho, Picone et al. 2014), who suggest that centriole amplification itself increases Rac1 activity. The Rho family GTPases Cdc42 and Rac1 also control cytoskeletal dynamics and cell movement via signaling to the Arp2/3 complex through their downstream effectors N-WASP and WAVE. While Plk4’s effect on cell motility and invasion is likely complex and regulated at several levels, we show that Plk4 phosphorylates the key Arp2/3 complex member Arp2. Furthermore, a DN-Rac1 construct does not abrogate Plk4’s promotion of cell migration, further supporting a more direct role for Plk4 in regulating actin polymerization through the Arp2/3 complex.
Chapter 6: Discussion

6.1 Overview of Results

At the outset of the experiments described in this thesis we had limited understanding of the role of Plk4 in cancer cell motility and invasion, and of the mechanisms that drive Plk4-related tumour progression. In addition, it was unknown whether Plk4 regulated tumour progression in vivo. Using both MEFs and cancer cells I showed the specific Plk4-dependence of cell spreading, protrusion formation, migration and invasion. To then elucidate the potential mechanisms involved I used a BioID screen searching for Plk4 interactors, and identified members of the Arp2/3 complex; I went on to confirm physical and functional interaction with Arp2 in mediating Plk4-driven cancer cell migration. In a flank xenograft murine model, Plk4 depleted breast cancer cells showed reduced local invasion and lung metastasis. In conjunction with suppression of tumour progression, Plk4 depletion induced reversion to an epithelial phenotype in poorly differentiated breast cancer cells. These results validate Plk4 as a therapeutic target in cancer patients, and reveal a new function for Plk4 in regulating actin polymerization through the Arp2/3 complex. The data presented in this thesis therefore provide new insights into the function of Plk4 and its relevance to carcinoma progression. Nevertheless, regulation of actin cytoskeletal dynamics is a complicated process with involvement of multiple signalling pathways and molecular mechanisms, and in particular the relationship between Plk4 and the Rho GTPases remains to be explored. In the discussion below I will attempt to put into context some of these findings and suggest further experiments that will augment our understanding of Plk4 and its relevance to carcinogenesis and carcinoma progression.
6.2 Plk4 and cancer progression

Death from solid tumors is most commonly related to metastases, making prevention and control of tumor invasion a major therapeutic focus. Clinical studies indicate that Plk4 functions as a potential oncogene in established cancers, particularly of the breast, colon, and pancreas (Macmillan, Hudson et al. 2001, Ringner, Fredlund et al. 2011, Mason, Lin et al. 2014, Li, Dai et al. 2016, Hedley personal communication). Increased Plk4 activity causes centrosomal amplification and multipolar spindle formation, which can lead to mitotic errors and chromosomal instability. In keeping with this, some Plk4 overexpressing murine models in a p53 null background show evidence of aneuploidy and advanced tumor formation, namely lymphomas, sarcomas and skin squamous cell carcinomas (Coelho, Bury et al. 2015, Sercin, Larsimont et al. 2016). In addition to the roles of Plk4 in tumourigenesis, emerging evidence suggests that increased Plk4 levels in established malignancy may promote tumour progression by increasing cell motility and invasion in vitro (Godinho, Picone et al. 2014, Rosario, Kazazian et al. 2014). Here I show that Plk4 does in fact promote cancer cell motility and invasion in vitro, and invasion and metastatic progression in vivo, which had not been previously demonstrated. I used a MDA-MB-231 breast cancer xenograft model, with subcutaneous injection of cells transduced with Plk4 shRNAs or control RFP shRNA into the flanks of immunosuppressed mice. While the effect of depletion of Plk4 had a modest effect on tumour growth, consistent with the recent demonstration by Wong et al. that cancer cells continue to proliferate despite Plk4 inhibition and progressive loss of centrioles, albeit at a reduced rate (Wong, Anzola et al. 2015), Plk4 depletion resulted in significant suppression of locally aggressive behaviour and metastatic progression compared to control. There was no demonstrated change seen in Plks1-3 expression in the xenografts, demonstrating specificity of
the invasive and metastatic phenotypes to Plk4. This differs from the results shown by Mason and colleagues (Mason, Lin et al. 2014), who used a dual Plk4 and Aurora B kinase inhibitor, CFI-400945. While CFI-400945 did not have significant inhibitory activity towards Plks1-3, it did show significant activity against other kinases, including TRKA, TRKB, Tie2/TEK, and Aurora B kinase, *in vitro* and in cells. In particular, treatment with CFI-400945 resulted in cytokinesis failure and accumulation of polyploid cells, a phenotype ascribed to Aurora B inhibition. As Aurora B inhibitors show efficient antitumour activity in and of themselves, with several now in Phase I or II clinical trials ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)), it is likely that some of the reduction in xenograft size seen with CFI-400945 could be attributable to Aurora B kinase inhibition. This presents an important caveat for the application of Plk4 inhibitors in cancer therapy, where greater utility may be found with the goal of suppression of invasion and metastasis rather than growth suppression, as our data would appear to indicate.

In keeping with the reduction in migration/invasion *in vitro* and local invasion and metastatic potential with Plk4 depletion in our xenograft model, the phenotypic features and expression profile in cancer cells and xenografts with Plk4 depletion was consistent with a shift from a mesenchymal to epithelial (MET) phenotype. Induction of EMT is considered a key step in epithelial cell-derived cancer metastasis and progression (Thiery 2002), with evidence indicating that cancer cells acquire expression of mesenchymal markers and activate the EMT program in promoting epithelial cell migration and increasing the metastatic and invasive potential of these cells (Kang and Massague 2004, Yang, Mani et al. 2004, Huber, Kraut et al. 2005, Guarino 2007, Tiwari, Gheldof et al. 2012). Here I showed that HeLa cells, which express Twist and thereby have a program for EMT in place, when depleted of Plk4, gained expression of the epithelial marker E-cadherin and lost expression of fibronectin and other mesenchymal markers. Similarly,
Plk4-depleted MDA-MB-231 xenografts displayed a more "epithelial" gene expression pattern than did control tumours in the majority of genes assayed, in keeping with the phenotype of decreased tumour invasiveness. However, how EMT is induced during Plk4-related cancer progression remains unexplored and is an area for further study. It is possible that activation of signalling pathways that promote EMT are also regulating expression of Plk4; for example, NF-kappaB signalling, which has been shown to be one of the important regulators of the EMT program (Huber, Azoitei et al. 2004), regulates expression of Plk4 (Ledoux, Sellier et al. 2013). It is also possible that Plk4 may be regulating one of the transcription factors capable of repressing E-cadherin, and this is another area of possible study, where previous studies show that Plk4 is able to phosphorylate transcription factors, namely the bHLH transcription factor Hand1 to promote trophoblast differentiation (Martindill, Risebro et al. 2007). Finally, Plk4 mediated centriole loss and amplification induces p53 expression, and p53 in turn represses EMT by activating expression of various microRNAs to maintain an epithelial phenotype (Chang, Chao et al. 2011), another potential mechanism that can be explored in future experiments. Further characterization of the mechanisms by which Plk4 contributes to EMT could lead to new therapeutic approaches for antimetastatic cancer treatments.

Overall, these findings deepen our appreciation of Plk4 as a potential therapeutic target in cancer treatment. Nevertheless, Plk4 has also been shown to be haploinsufficient for tumour formation in hepatocytes: Elderly Plk4+/− mice have a 50% incidence of spontaneous tumour formation, the majority being hepatocellular carcinomas (Ko, 2005) and human HCC specimens shows loss of heterozygosity at the Plk4 locus in approximately 50% of patients, with an accompanying decrease in Plk4 expression in the same patients’ tumours (Rosario, 2010). Reduced Plk4 expression has also been associated with worse survival in HCC patients.
In addition, we have shown that Plk4 depletion also suppresses motility in normal cells (MEF, HEK293T) (Chapter 3; (Rosario, Kazazian et al. 2015)), suggesting potential for altering neutrophil, macrophage and lymphocyte motility, and compromise of immune cell function and wound healing. This raises some concern with the application of Plk4 inhibitors, as the clinical sequelae of long-term use remains unknown and judicious selection of patients will be an important consideration.

6.3 Plk4, centrioles and actin cytoskeleton dynamics

Centrosomes are the major microtubule-organizing centres of animal cells, participating in a number of key cellular processes such as cell division. Extra centrosomes are a common feature of cancer cells, and evidence has been generated to imply a causative role of centrosomal amplification in carcinoma development and progression. One proposed mechanism is through spindle anomalies and aneuploidy. However, clustering at the spindle poles facilitates bipolar division even in the presence of centrosomal amplification (Ring, Hubble et al. 1982, Quintyne, Reing et al. 2005, Ganem, Godinho et al. 2009). Pellman and colleagues have proposed another potential mechanism through which supernumerary centrioles may facilitate carcinogenesis, namely through enhanced cell invasion. In their model, centriole number was manipulated by altering Plk4 levels or through cytokinesis defects. With the latter method, it is plausible, though not explored in the study, that there was Plk4 overexpressed at the multiple centrioles due to the resultant centriole amplification (Godinho, Picone et al. 2014). Denu and colleagues have also described the acquisition of high grade features in cells following manipulations that increased cellular centriole number (Denu, Zasadil et al. 2016). In consideration of the possibility that Plk4-induced centriolar amplification might mediate the motility-related phenotypes we observed here, we compared the effect of Plk4 manipulation on change in centriole number and
change in migration/invasion when compared to control, and found them to differ significantly in magnitude. We more closely evaluated migration in our various cancer cell lines, as the wound healing assay was highly reproducible and quantifiable, and could not find correlation between magnitude of centriole amplification or loss, and the change in wound healing migration. It was true, however, that the direction of the change in centriole number typically correlated with the direction of change in motility. For instance, while stable upregulation of Plk4 in U2OS cells using 0.01µg/ml tetracycline had minimal impact on centriole number, it significantly increased wound healing with a magnitude of effect similar to that seen with higher doses of tetracycline and larger amplitudes of centriole amplification. This suggested that while Plk4’s effect on cell motility and invasion is likely complex and regulated at several levels, and Plk4 levels are intimately tied to centriole number, there may be an additional Plk4 substrate that is mediating its effect on migration/invasion, at least in part. Indeed, I showed that Plk4 interacts with and phosphorylates the key Arp2/3 complex member Arp2. Also, treatment of MDA-MB-231 cells with the specific Plk4 inhibitor, centrinone B, over a short time course that does not impact centriole number, does reduce migration, further indicating that Plk4 can affect cancer cell motility independent of changes in centriole number. Further experiments can focus on determining a dose-effect response of centrinone on migration.

Identification of Arp2 as a novel Plk4 interactor began with an unbiased screen using BioID, and culminated in evidence that the Arp2/3 complex is a downstream effector of Plk4 in promoting cancer cell motility. Altered expression of one or more of the seven Arp2/3 complex subunits has been shown in several types of human epithelial malignancy, including gastric, lung and colorectal cancer (Kaneda, Kaminishi et al. 2004, Otsubo, Iwaya et al. 2004, Semba, Iwaya et al. 2006, Zheng, Zheng et al. 2008, Sun, Shang et al. 2014). In particular, high Arp2
expression correlates with aggressive behavior and poor prognosis (Otsubo, Iwaya et al. 2004, Semba, Iwaya et al. 2006, Zheng, Zheng et al. 2008, Sun, Shang et al. 2014). In keeping with this, silencing of Arp2 reduces migration in pancreatic and gastric cancer cell lines (Rauhala, Teppo et al. 2013, Sun, Shang et al. 2014). However, not all cell motility is dependent on Arp2/3 activity (Wu, Asokan et al. 2012), as illustrated by the insensitivity of A2780 cancer cell migration to CK-666(Paul, Allen et al. 2015). The dependence of Plk4-induced motility phenotypes on Arp2 and Arp2/3 complex activity was therefore important to establish; this was observed in spreading HeLa cells and migrating U2OS cells. Therefore, in considering the rational selection of patients for Plk4 inhibitor therapy, determination of Arp2 status may be useful.

Until recently the Arp2/3 complex was thought to be primarily regulated by nucleation promoting factors (NPFs) including the WASP/WAVE proteins. However, recent evidence indicates that it is also phosphorylated by serine-threonine kinases, and that this phosphorylation is necessary to activate the Arp2/3 complex to promote actin nucleation and lamellipodia formation (LeClaire, Baumgartner et al. 2008). The Nck-interacting kinase (NIK) is the first Arp2 interactor that has been identified to directly bind and phosphorylate Arp2 at T237+T238, leading to increased actin nucleating activity of the Arp2/3 complex and promoting plasma membrane protrusion in response to epidermal growth factor (LeClaire, Rana et al. 2015). I showed that Plk4 also phosphorylates Arp2 in an in vitro kinase assay, and this appears to be specific to the T237 and T238 residues. Phosphorylation of Arp2 by Plk4 suggests that Plk4’s effect on cell motility and invasion may be mediated by the increased actin nucleating activity of the Arp2/3 complex. While I showed that Plk4 phosphorylates Arp2, Plk4 promotes actin rearrangement towards a wound, and while previous studies show that phosphorylated Arp2 is
more effective in promoting Arp2/3 complex based actin polymerization in pyrene labeled actin assays, I have not directly shown the effect of Plk4 in actin polymerization in vitro and this can be an additional area of study. A further area for exploration is to determine the relative contribution of Plk4-based increased Rac1 (or Cdc42) activation, and thereby activation of WASP/WAVE vs. Arp2 phosphorylation on Arp2/3 complex activity. This can be done by using dominant negative constructs or treating cells with NSC23766 to antagonize Rac1 signalling, and then assessing Plk4’s effect on Arp2/3 complex based actin polymerization in pyrene labeled actin assays.

While we propose that Plk4, which transiently localizes to cell protrusions following a stimulus to migrate, interacts with Arp2 at the cell periphery to stimulate actin nucleation, the Arp2/3 complex has also been shown in some cases to localize to the centrosome (Hubert, Vandekerckhove et al. 2011). In fact, several actin-binding proteins are present at the centrosome, including cortactin, CapG and the Arp2/3 activator WASH (Wang, Chen et al. 2008, Hubert, Van Impe et al. 2009, Monfregola, Napolitano et al. 2010), generally involved in regulating the actin cytoskeleton during cell division. Members of the Arp2/3 complex, specifically ARPC1B, have been shown to transiently localize to the pericentriolar material of interphase cells in about 10% of HEK293T cells, but not in MDA-MB-231s. These authors hypothesized that the complex is recruited to the centrosome through direct binding of the ARPC1B subunit to centrosomal Exo70 (a subunit of the exocyst complex), also responsible for recruitment of the complex to the lamellipodium (Hubert, Vandekerckhove et al. 2011). They proposed that the centrosome transiently recruits Arp2/3 to perform processes such as centrosome separation prior to mitotic entry or actin-based centrosome reorientation toward the leading edge in migrating cells, although this was not shown, and effect on cell migration was
It is possible that Plk4 may interact with Arp2/3 at the centrosome, although Arp2/3 pericentriolar matrix localization was present in only one cell type assessed. In contrast, we did show that the effect of Plk4 on cell migration was consistent regardless of cell type, including MDA-MB-231 cells, which did not localize the Arp2/3 complex to the centrosome in their study (Hubert, Vandekerckhove et al. 2011), and we did confirm localization of Arp2 and Plk4 to the lamellipodial front, supporting our proposed model that they interact at the cell leading edge to promote cell protrusion formation and migration. Nevertheless, we do know that the centrosome is intimately tied to the directionality of cell movement, and several centrosomal proteins have been shown to affect cell motility, including Par6gamma, AuroraA, CEP55, CEP70, AKAP350, ninein-like protein and CEP192, and in some cases to correlate with carcinoma progression and metastasis (Guan, Wang et al. 2007, Shi, Liu et al. 2012, Dormoy, Tormanen et al. 2013, O'Rourke, Gomez-Ferreria et al. 2014, Liu, Wang et al. 2015, Tonucci, Hidalgo et al. 2015, Zhang, Niu et al. 2016). In particular, CEP192, which is a centrosomal scaffold protein that recruits Plk4 to the centriole and regulates centrosomal microtubule nucleation, when depleted negatively impacts cell motility and invasion (Godinho, Picone et al. 2014, O'Rourke, Gomez-Ferreria et al. 2014). In the study by Pellman’s group, depletion of CEP192 inhibited Rac1 activation and restored normal cell–cell adhesion among cells with centrosome amplification, suppressing the invasive phenotype in cells with extra centrosomes, suggesting that increased centrosomal microtubule nucleation in cells with extra centrosomes triggers invasion (Godinho, Picone et al. 2014). Nevertheless, it remains unclear how dynamic microtubules activate Rac1, and CEP192 in some studies was required for Plk4 localization, raising several questions regarding the contribution of centrosomes and microtubules vs. the actin cytoskeleton to Plk4-mediated cell migration/invasion. The microtubular and actin
cytoskeletons have complex interactions in motile cells, with direct and indirect means of coregulation, and previous work from our laboratory suggests that Plk4 may be transported to cell protrusions via the plus end of microtubules and delivered to zones of actin polymerization. It is thus difficult to precisely delineate the relative contributions of the microtubule and actin cytoskeletons in Plk4-mediated cell migration/invasion. Disrupting the microtubule and actin cytoskeletons using nocodazole or colchicine (microtubules) or cytochalasin-D (actin) may help answer this question, although interpretation may be difficult due to the codependence in cellulo. Here we show that Plk4 does in fact affect the actin cytoskeleton by interacting with and phosphorylating the Arp2/3 complex member Arp2.

6.4 Plk4 and the RhoGTPases

Plk4 has previously been shown to increase Rac1 activity (Brashavitskaya, Kazazian et al. 2013, Godinho, Picone et al. 2014); the latter group suggested that this mediates the associated increase in cellular invasiveness, and is triggered by centriole amplification and subsequent increased microtubule nucleation. In our system, while transfecting cells with dominant negative Rac1 modestly reduced cell migration as expected, we found in our wound healing assays that overexpression of Plk4 using a tetracycline inducible system in U2OS cells transfected with DNRac1 nevertheless resulted in increased wound healing/migration similar to what we saw with Plk4 overexpression alone. In addition, we did not detect increased active Rac1 with tetracycline-induced Plk4 overexpression in these cells. Thus a Plk4 mediated increase in Rac1 activity, such as that shown by Pellman’s group in MCF10A cells (Godinho, Picone et al. 2014) could not fully explain the Plk4-induced enhancement of motility in our assays. Our findings instead suggest an alternate mechanism, a more direct effect of Plk4 on the actin cytoskeleton, as discussed above. Nevertheless, the potential relationship between Plk4/centrosome amplification
and Rac1-mediated invasion is an important area for further study. Pellman and colleagues propose that increased centriole-mediated microtubule nucleation is responsible for increased active Rac1 in cells (Godinho, Picone et al. 2014). Our group has previously shown that Plk4 can phosphorylate the RhoA GEF Ect2, and has demonstrated dependence of Plk4-induced activation of RhoA on Ect2 (Rosario, Ko et al. 2010). However, while RhoA is not required for MTOC polarization or for formation of protrusions (Etienne-Manneville and Hall 2001) and we did not investigate this further, it is possible that Plk4 can, in a similar fashion, interact with other RhoGTPase GEFs and/or GAPs to induce activation of Rac1 or Cdc42, suggesting another mechanism for Plk4’s effect on RhoGTPase activity. To explore this further, our group has interrogated a library of 145 GEFs, GAPs and GDIs for the core consensus phosphorylation motif for Plk4 (Leung, Ho et al. 2007, Sillibourne, Tack et al. 2010), and 12 further potential substrates for Plk4 phosphorylation were identified (ARHGEF1, PREX2, ALS2, PLEKHG6, RGNEF, TRIO, DOCK2, DOCK3 and DOCK5–8). These are currently being investigated for interaction with Plk4 in regulating motility.

In our investigation of the effect of Plk4 on cell motility, we noted defective polarity in migrating fibroblasts and cancer cells, where there was impaired reorientation of the MTOC/Golgi relative to the nucleus when cells were stimulated to migrate. This is one of the early events required for directional migration, with asymmetric reorganization of cell components in order to acquire a front-rear/migratory polarity, where the nucleus moves rearward and the centrosome and Golgi complex relocate to the front of the cells. This polarized organization is required for directional secretion of regulatory proteins towards the cell leading edge and wound healing migration (Yadav, Puri et al. 2009, Etienne-Manneville 2013). Cdc42 activation at the leading edge is required for centrosome and Golgi reorientation, where the
nucleus moves backwards while the centrosome is kept in its central position by a dynein- and microtubule-dependent process (Gomes, Jani et al. 2005). In addition, Cdc42 is essential for the formation of filopodial protrusions early after wounding, while Rac1 is essential for both the development and the maintenance of protrusions during migration (Ridley, Paterson et al. 1992, Etienne-Manneville and Hall 2001, Wu, Frey et al. 2009). We therefore explored Cdc42 activation in relation to Plk4, and found that in Plk4-depleted HeLa cells there was relatively less activated Cdc42. Of note, Cdc42 also induces actin polymerization and plays a role in activating the Arp2/3 complex via the nucleation promoting factor N-WASP (Rohatgi, Ma et al. 1999).

However, in our wound healing migration assays we were not able to show an effect of transfection with DNCdc42T17N on U2OS cell migration, conceivably reflecting the cooperative nature of Rho GTPase function during cell migration. This may also be reflective of the fact that the contribution of individual RhoGTPases to regulation of cytoskeletal organization and cell migration can be cell type dependent. For example, while the loss of Cdc42 function in primary mouse fibroblasts has been reported to impair migration speed (Yang, Wang et al. 2006), it has no effect on fibroblastoid cells (Czuchra, Wu et al. 2005) or Drosophila peripheral glial cells (Sepp and Auld 2003), and actually enhances the speed of Drosophila hemocytes (Stramer, Wood et al. 2005) and macrophages (Allen, Zicha et al. 1998). This may reflect functional specialization within a cell, which is likely to be dictated by the relative abundance of the RhoGTPases and the availability of specific downstream effectors; therefore, loss of function of a particular GTPase can result in a more severe phenotype for one cell type than for another. Additional studies will be required to elucidate whether/how Plk4 participates in Cdc42 and Rac1 activation to promote cell migration, and the relative contribution of the RhoGTPases vs. activation of the Arp2/3 complex in Plk4-mediated cancer cell migration/invasion. In addition,
similar to the findings of Pellman’s group and others, disruption of the microtubule cytoskeleton affects both Cdc42 and Rac1 activation (Gauthier-Rouviere, Vignal et al. 1998, Waterman-Storer and Salmon 1999, Waterman-Storer, Worthylake et al. 1999), further highlighting the interdependence of the actin and microtubule cytoskeletons and complicating study of how precisely Plk4, centrosomes, microtubules and the actin cytoskeleton regulate cancer cell invasion.

6.5 Centrioles and cancer

The centrosome consists of a pair of centrioles surrounded by pericentriolar material, a complex proteinaceous structure in which the centrioles are embedded. In differentiated cells, the mother centriole also functions as the basal body which assembles primary cilia. During the cell cycle, centrosomes duplicate once during S phase to direct assembly of a bipolar mitotic spindle, although work in Drosophila and mammalian cells has shown that cells without centrioles can also assemble bipolar spindles (Khodjakov, Cole et al. 2000, Bettencourt-Dias, Rodrigues-Martins et al. 2005, Basto, Lau et al. 2006). Deregulation of this process may result in an altered centrosome complement and formation of monopolar or multipolar mitotic spindles, and this can lead to chromosomal instability and aneuploidy (Ganem, Godinho et al. 2009, Sir, Putz et al. 2013). Boveri’s proposal that an increased number of centrosomes causes cancer, based on the idea that centrosome amplification causes improper chromosome segregation during mitosis and thereby triggers malignancy, has been a source of controversy over the past century (reviewed in (Boveri 2008)). Many studies have shown that centrosome abnormalities are a common feature of human cancer (reviewed in (Chan 2011)), including solid tumours such as breast, colorectal, pancreatic, ovarian and prostate carcinomas as well as hematological malignancies such as lymphomas, leukemias and multiple myeloma (Lingle, Lutz et al. 1998, Pihan, Purohit et al. 2006).
In most human cancers, centrosome amplification has been associated with high-grade tumours and poor prognosis, and in breast, prostate and head and neck carcinomas specifically it has been associated with lymph node positivity and metastasis, suggesting a potential role in carcinoma progression and highlighting its potential as a biomarker for advanced disease (Pihan, Purohit et al. 2001, D'Assoro, Barrett et al. 2002, Reiter, Gais et al. 2009). Centrosomal defects in human cancers are classified as structural or numerical. Numerical defects, such as centrosome amplification, are the most frequently described in cancer. Centrosome amplification can result from several different mechanisms, including cytokinesis failure, cell-cell fusion, overduplication of centrioles and de novo centriole assembly. There are also numerous pathways and proteins including tumour suppressors and oncogenes that are associated with centrosome amplification and cancer, including CDK-cyclin complexes, p53, Aurora A kinase, myc and many others in addition to the master regulator of centriole duplication Plk4 (Fukasawa 2007).

While overexpression of centriole proteins like Plk4 and Sas-6 can result in centrosome amplification (Peel, Stevens et al. 2007), deregulation of subunits of the ubiquitin ligase complexes such as βTrCP leading to stabilization of Plk4 (Cunha-Ferreira, Bento et al. 2009, Rogers, Rusan et al. 2009) or overexpression of USP33 resulting in increased deubiquitinated CP110 (Li, D'Angiolella et al. 2013), also lead to centrosome amplification. Centriolar proteins can also be regulated at the transcriptional level. For example, the HPV16 E7 oncoprotein activates the Plk4 promoter to upregulate Plk4 mRNA expression and induce centrosome amplification, while p53 negatively regulates Plk4 levels by recruitment of HDAC (histone deacetylase) transcriptional repressors to the Plk4 promoter (Li, Tan et al. 2005). Extra
centrosomes themselves can induce p53 stabilization (Holland, Fachinetti et al. 2012), to maintain the normal centrosome complement in cells. Loss of other tumour suppressors, such as BRCA1 and BRCA2, can also result in centrosome amplification through increased expression of γ-tubulin and cell division errors, respectively (Tutt, Gabriel et al. 1999, Starita, Machida et al. 2004). Cytokinesis failure can also result in centrosome amplification and generate tetraploid p53 null cells that induce tumourigenesis in murine models (Fujiwara, Bandi et al. 2005, Ganem, Godinho et al. 2009). However, it is clear that centrosome amplification is detrimental to cells. Tetraploid cells spontaneously lose their extra centrosomes over time in culture (Ganem, Godinho et al. 2009) and induction of centrosome amplification with Plk4 overexpression negatively impacts the fitness of mammalian cells and tissues, and other permissive conditions such as p53 loss need to be present for continued proliferation (Marthiens, Rujano et al. 2013, Lambrus, Uetake et al. 2015, Vitre, Holland et al. 2015, Sercin, Larsimont et al. 2016). Some cells, including hepatocytes, are however able to tolerate extra centrosomes (Faggioli, Vezzoni et al. 2011), and cancer cells have exploited compensatory mechanisms to manage the effects of centrosome amplification and form bipolar spindles. Centrosome clustering to suppress multipolar mitoses is one such mechanism (Quintyne, Reing et al. 2005, Kwon, Godinho et al. 2008). This raises the question regarding how chromosomal instability and aneuploidy can be a consequence of centrosome amplification if the majority of spindles are bipolar due to compensatory centrosome clustering or loss of extra centrosomes. One possibility is the establishment of merotelic attachments, in which a single kinetochore is attached to both spindle poles, which can generate lagging chromosomes during mitosis and consequently aneuploidy (Ganem, Godinho et al. 2009). Perturbing spindle bipolarity, resulting in cell apoptosis, has therefore emerged as a promising approach to selectively target tumour cells that contain extra
centrosomes; in this light, the use of HSET, a kinesin motor, inhibitors can be considered (Kwon, Godinho et al. 2008).

The consequences of Plk4 overexpression and centrosome amplification have been studied in *Drosophila* and murine models. In both species, the presence of extra centrosomes was poorly tolerated (Basto, Brunk et al. 2008, Marthiens, Rujano et al. 2013, Vitre, Holland et al. 2015, Sercin, Larsimont et al. 2016). While ~60% of *Sak* overexpressing *Drosophila* embryos die during embryogenesis, the remainder continue to develop without evidence of genetic instability or tumourigenesis; however, when *Sak* overexpressing larval brain cells with centrosome amplification are transplanted into the abdomen of wildtype hosts they generate metastatic tumours (Basto, Brunk et al. 2008). In contrast to *Drosophila*, Plk4 overexpression in the murine brain does result in aneuploidy, where inefficient centrosome clustering generates multipolar mitoses, and aneuploid neuronal stem cells undergo apoptosis leading to microcephaly without tumourigenesis (Marthiens, Rujano et al. 2013). In the context of p53 dysfunction, increased Plk4 expression and resultant centrosome amplification contribute to aneuploidy or hyperproliferation, in a manner which appears tissue dependent, and tumourigenesis (Coelho, Bury et al. 2015, Sercin, Larsimont et al. 2016), although other studies did not find evidence of tumourigenesis with Plk4 overexpression and centrosome amplification (Kulukian, Holland et al. 2015, Vitre, Holland et al. 2015). It is clear that not all tissues respond in the same way to Plk4 overexpression, centrosome amplification and aneuploidy. This is reminiscent of reducing the levels of the mitotic kinesin CENP-E, which causes aneuploidy, but generates tumours in only some tissues and acts as a inhibitor of tumourigenesis in others (Weaver, Silk et al. 2007). It is also likely that for a given phenotype the precise level of Plk4 overexpression or depletion, and the cellular and tissue context, are very important. This further highlights the importance of
gaining a better understanding of the relationship between Plk4 activity, stability and downstream functional effects, which as discussed below appears complex and is as of yet incompletely understood.

6.6 Plk4 inhibition as a strategy for treatment of aggressive carcinomas

The mammalian polo family of kinases comprises five members, four of which can act as haploinsufficient tumour suppressors (Plk1-4), and two of which are also regarded as potentially oncogenic (Plk1, Plk4) (reviewed in (Kazazian, Brashavitskaya et al. 2015)). Plk1 plays numerous and disparate roles throughout the cell cycle, including facilitation of centriole maturation, cytokinesis, mitotic entry, and the DNA damage response. In keeping with the promotion of cell cycle progression by Plk1 and its increased expression in human cancers, Plk1 inhibitors have been shown to suppress cancer cell proliferation and tumour growth (reviewed in (Kazazian, Brashavitskaya et al. 2015)). Targeting of Plk1 in clinical cancer management is grounded in an extensive preclinical experience and has only recently reached phase III trials, as an adjunct to conventional cytotoxic chemotherapy. Plk4 has more recently emerged as a therapeutic target of potential interest, based largely on its increased expression in breast cancer patients who have an adverse prognosis. Though in some ways similar to Plk1 in localization and cellular function, Plk4 has sequence and structural properties that underlie its distinct array of molecular interactions and functions (Cheng, Lowe et al. 2003, Elia, Rellos et al. 2003, Lowery, Lim et al. 2005, Slevin, Nye et al. 2012, Zitouni, Nabais et al. 2014). Up to the present time, its appeal as a therapeutic target has been founded largely on the notion that its overexpression is responsible for pathologic centrosomal amplification that is relatively unique to cancer cells.

The synthetic ATP-competitive inhibitor, CFI-400945, was developed as a selective Plk4 inhibitor and was shown to suppress breast and colorectal cancer cell proliferation in vitro and
xenograft growth in vivo, most clearly in PTEN mutant cells (Laufer, Ng et al. 2014, Mason, Lin et al. 2014). With this preclinical basis, CFI-400945 has entered phase I clinical testing in humans; however, along with inhibiting Plk4, CFI-400945 also inhibits Aurora B kinase both in vitro and in vivo, complicating the interpretation of its effects (Holland and Cleveland 2014, Mason, Lin et al. 2014). The authors and others speculate that Aurora B inhibition accounts for the impairment of cytokinesis observed in cell culture, and that the antiproliferative effect of CFI-400945 may be ascribed largely to its inhibition of Aurora B kinase, suggesting that inhibition of Plk4 activity might prove useful in cancer therapy only in combination with other strategies.

Another conundrum is the bimodal effect of CFI-400945 on centriole number, as lower doses of the inhibitor fail to suppress centriole duplication, the canonical function of Plk4, and in fact drive an increase in centriole number. This is reminiscent of stable overexpression of kinase dead Plk4, which causes centriole overduplication in the presence of endogenous wildtype Plk4, as kinase dead Plk4 is unable to trans-autophosphorylate and destabilize wildtype Plk4 (Guderian, Westendorf et al. 2010). This phenomenon also recalls the phenotype of Plk4+/− MEFs, which display a modest increase in centrosome number, which our group attributed to defective cytokinesis. It is possible that lower doses of CFI-400945 that partially inhibit Plk4 are in fact stabilizing and increasing the total amount of active Plk4 (Holland and Cleveland 2014), which is concerning in the light of the findings from my work on breast cancer xenografts, where Plk4 promotes carcinoma progression as well as aneuploidy and tumourigenesis (Coelho, Bury et al. 2015, Sercin, Larsimont et al. 2016). It is therefore going to be important to identify and maintain a narrow effective dose of inhibition in the tumour itself, to avoid centrosome amplification and tumourigenesis as well as suppress tumour progression. Plk4 is also a
haploinsufficient tumour suppressor for hepatocellular carcinoma development in mice and likely in humans (Ko, Rosario et al. 2005, Rosario, Ko et al. 2010). Moreover, the progression of spontaneous lymphomas in Plk4 heterozygous mice is accelerated by p53 depletion (Ko, Rosario et al. 2005). This raises a concern about the potential for development of second malignancies with prolonged Plk4 inhibitor therapy. Therefore, while there is pressing interest in Plk4 as a therapeutic target, the clinical sequelae, particularly in relation to hepatocellular carcinoma and carcinoma progression, remain unknown.

Recently, Oegema and colleagues used a highly specific Plk4 inhibitor, centrinone, to interrogate the role of centrioles in cancer cell proliferation (Wong, Anzola et al. 2015). Unlike nontransformed cells, cancer cells tolerate complete inhibition of Plk4, and though they lacked centrioles, proliferated at a new lower but sustainable rate. Following removal of centrinone, the cells returned to their basal centriole profile and proliferative rate. Therefore, while centrinone caused apoptotic cell death in normal cells, cancer cells continued to proliferate (Wong, Anzola et al. 2015), indicating potential difficulties with the therapeutic window if used clinically. Prior work using untransformed mammalian cells had showed that transient centrosome removal, with microsurgery and laser ablation, did not block cell cycle progression through G1, and these cells were subsequently able to assemble centrioles de novo. (Uetake, Loncarek et al. 2007). The advent of specific Plk4 inhibition with centrinone allowed for analysis of the effect of multigenerational centrosome depletion, highlighting the differences between transformed and nontransformed cells in their tolerance of an acentriolar state. In untransformed cells, centrosome loss led to cell cycle arrest in G1, in a p53 dependent manner, which was not a consequence of DNA damage, extended mitotic duration, aneuploidy/chromosome missegregation, stress, or Hippo signaling. This work has therefore identified a centrosome loss
sensor that arrests cells in G1 in a p53 dependent manner. In general, cancer cell lines, a large number of which have mutations or deleted/degraded p53, are able to bypass this p53 dependent arrest. Of the 21 cell lines treated with centrinone that continued to proliferate in the absence of centrosomes, 12 had mutations in or suppressed expression of p53 (the other common deletion/silencing was in the cyclin dependent kinase inhibitor 2a (Cdkn2a), which acts as a negative regulator of proliferation by inhibiting Cdk4 and Cdk6), which suggested that the arrest is p53- dependent. Consistent with this, increased protein levels of p53 and its downstream effector p21 were observed after centrosome depletion. This study revealed that centrosomes are essential for the proliferation of normal mammalian cells, in contrast to what is observed in Drosophila (Basto, Lau et al. 2006).

The differential effect of centrosome loss on normal cells and cancer cells suggests that Plk4 inhibition may have its greatest effects on non-cancer processes, including embryogenesis (Hudson, Kozarova et al. 2001), fertility (Khire, Vizuet et al. 2015, McCoy, Demko et al. 2015), primary cilium formation, brain health and body growth, (Martin, Ahmad et al. 2014), as well as processes such as wound healing, or immune cell function that depend on cell migration but have not been explored in the context of Plk4. Plk4’s importance in embryogenesis is evident in that the Plk4^{−/−} genotype is early embryonic lethal in mice, while Plk4^{+/−} mice develop normally but exhibit increased rates of liver and lung cancer (Hudson, Kozarova et al. 2001, Ko, Rosario et al. 2005). Although Plk4^{+/−} MEFs were shown by our group to display centrosome amplification, also seen with low doses of the Plk4 inhibitor CFI-400945 (Mason, Lin et al. 2014), and increased levels of tetraploidy (Rosario, Ko et al. 2010), other groups did not demonstrate centrosome amplification or aneuploidy in these MEFs when the cells were grown under different culture conditions (Holland, Fachinetti et al. 2012). Additionally, centrosomal Plk4
protein levels were unchanged, despite a 50% decrease in transcript levels in Plk4+/− cells, indicating that Plk4 protein at the centriole is more stable and resistant to depletion and that Plk4 regulates its own protein stability through autophosphorylation (Holland, Lan et al. 2010).

Others have studied the effects of more profound Plk4 depletion. In cells derived from individuals with Plk4 mutations, a decrease of approximately 75% in transcript levels impairs protein function and results in impaired centriole duplication with Plk4 autoregulation failing to compensate, but the protein having sufficient enzyme activity to prevent embryonic lethality. Plk4 mutations are seen in individuals with microcephaly and short stature, comparable to the phenotypes reported with mutations in several centriolar biogenesis genes, including CENPJ/CPAP, STIL, CEP135, CEP152 and CEP63 (Bond, Roberts et al. 2005, Kumar, Girimaji et al. 2009, Guernsey, Jiang et al. 2010, Sir, Barr et al. 2011, Hussain, Baig et al. 2012, Martin, Ahmad et al. 2014), indicating that Plk4 is important in neurogenesis and the developing brain. In the context of Plk4 mutations, additional developmental anomalies are also apparent, including generalized retinopathy. The retinal pathology observed by Martin and colleagues may have been a consequence of impaired mitosis in the eye or impaired cilia formation, as was seen in their zebrafish model (Martin, Ahmad et al. 2014). Ciliary dysfunction is seen with alterations in centriole number and Plk4 deregulation (Mahjoub and Stearns 2012); it is therefore possible that Plk4 inhibition can lead to ciliopathies, such as polycystic kidney disease, retinal degeneration, polydactyly, neural tube defects, respiratory illnesses and obesity (Waters and Beales 2011). Plk4 has also been implicated in fertility: A study by McCoy et al. (McCoy, Demko et al. 2015) showed that common variants spanning Plk4 are associated with mitotic-origin aneuploidy in human embryos, causing pregnancy loss. Moreover, it is likely that Plk4 inhibition will affect processes such as wound healing or immune cell function that depend on
normal cell migration, although this has not been studied to date. These findings emphasize the importance of a narrow therapeutic window for Plk4 inhibition in order to affect tumourigenesis or tumour progression while minimizing harmful adverse side effects.

Overall, it is essential to gain a better understanding of the molecular interactors that regulate Plk4 function in cancer cells, not only with respect to tumour growth, but also invasion/metastasis, as the effects on these phenotypes may differ. I showed that Plk4 plays a role in tumour progression by facilitating invasion and metastasis, and a possible role for Plk4 inhibition may be in the treatment of select patients with aggressive tumours to prevent tumour progression.
Chapter 7: Conclusions and Future Directions

7.1 Conclusions

Previous work had established Plk4 as a haploinsufficient tumour suppressor, while emerging evidence suggested a potentially oncogenic role for Plk4. The data I have gathered here demonstrate a role for Plk4 in facilitating tumour progression by enhancing cancer cell motility and invasion. I have shown the specific Plk4-dependence of cancer cell migration and invasion, as well as local invasion and metastasis of cancer xenografts. Furthermore, I demonstrate physical and functional interaction of Plk4 with the Arp2/3 complex member, Arp2, in mediating Plk4-driven cancer cell motility. In keeping with suppression of cancer invasion, Plk4 depletion reverts poorly differentiated HeLa cells and MDA-MB-231 breast cancer xenografts from a mesenchymal to an epithelial phenotype. Plk4 has been proposed as a therapeutic target in the treatment of advanced cancers based on its increased expression in primary human cancers, its facilitation of tumor growth in murine xenograft models, and the centrosomal amplification induced by its overexpression. However, a promising role for Plk4 inhibition may be in the treatment of select patients with aggressive tumours that are resistant to conventional systemic agents.
7.2 Future Directions

7.2.1. Upstream regulation of Plk4 activity in centriole duplication and motility.

One of the overarching questions in the Plk4 field is how Plk4 activity is regulated in cells. The vast majority of the work that has been done to date relates to its regulation of centriole duplication, using this function as an indicator of Plk4 activity. Plk4 is distinct from the other Plks in that it forms a homodimer and contains a triple PB structure (Guderian, Westendorf et al. 2010, Slevin, Nye et al. 2012). Plk4 generates its own phosphodegron, ultimately resulting in its degradation, by assembling as a homodimer and extensively trans-autophosphorylating its downstream regulatory element, which contains the conserved Slimb-binding motif. This then recruits the SCF$^{\text{Slimb/ßTrCP}}$ (Skp1/Cullin/F-box) E3 ubiquitin ligase complex to ubiquitinate Plk4, resulting in its degradation by the proteasome (Guderian, Westendorf et al. 2010, Holland, Lan et al. 2010, Cunha-Ferreira, Bento et al. 2013, Klebba, Buster et al. 2013). The E3 ubiquitin ligase Mind bomb (Mib1) was recently identified as a new interaction partner of Plk4. The E3 ligase activity of Mib1 triggers ubiquitylation of Plk4 at several sites, causing the formation of Lys11-, Lys29- and Lys48-ubiquitin linkages, thus restricting the abundance of Plk4 and counteracting centriole amplification induced by excess Plk4 (Cajanek, Glatter et al. 2015). Tight regulation of Plk4 in these ways is crucial to prevent the synthesis of extra daughter centrioles (Cunha-Ferreira, Rodrigues-Martins et al. 2009). In Drosophila, PP2A(Twins) counteracts fly Plk4/SAK autophosphorylation, thus stabilizing SAK and promoting centriole duplication (Brownlee, Klebba et al. 2011), although a corresponding phosphatase has not been identified in mammalian cells. Overall, it remains clear that our understanding of Plk4 regulation is incomplete and other regulatory mechanisms are likely to exist. A more complete understanding of Plk4 regulation is
important, particularly in view of its frequent “overexpression” in human cancers (Macmillan, Hudson et al. 2001, van de Vijver, He et al. 2002, Chng, Braggio et al. 2008, Mason, Lin et al. 2014) and the impact of Plk4 mutations causing microcephaly, growth failure and infertility (Harris, Weiss et al. 2011, Martin, Ahmad et al. 2014, McCoy, Demko et al. 2015). My work further highlights the importance of understanding Plk4 regulation as it relates to other Plk4 functions, most saliently the promotion of cell movement. Alternate upstream signaling pathways may exist, and be subject to different inputs, as compared to regulation of centriole duplication.

A candidate Plk4-interacting protein that may function to regulate Plk4 expression and is being investigated in our laboratory is FAM46C. FAM46C is a conserved protein, which we have shown functions as an endogenous inhibitor of Plk4 activity. FAM46C was initially identified in two separate yeast two-hybrid screens as a potential Plk4 interactor (Rual, Venkatesan et al. 2005, Murali, Pacifico et al. 2011), prompting our interest in this protein. Little is known about the function of FAM46C, although it has been suggested that FAM46C may be functionally involved with the Type 1 interferon response (Schoggins, Wilson et al. 2011), and FAM46C is recurrently mutated and inactivated in multiple myeloma in a pattern typical of a tumour suppressor gene (Boyd, Ross et al. 2011, Chapman, Lawrence et al. 2011, Bolli, Avet-Loiseau et al. 2014).

Upon FAM46C depletion, cells exhibit a centriole overduplication phenotype, similar to what is commonly observed with Plk4 overexpression, while FAM46C overexpression reduces the number of centrioles per cell (unpublished data). In an in vitro kinase assay, FAM46C suppresses Plk4 autophosphorylation, and when Flag-Plk4 and RFP-FAM46C are co-expressed in cells, there is a consistent increase in Flag-Plk4 WT protein expression in a dose-dependent
manner supporting the interpretation that FAM46C is inhibiting Plk4 kinase activity/autophosphorylation and thereby increasing stability of Plk4 (unpublished data). I used a MDA-MB-435 flank xenograft model in nude mice to investigate the potential regulation of Plk4 activity by FAM46C in vivo; MDA-MB-435 xenografts were utilized as they grow rapidly, although I found that they did not invade or metastasize prior to achieving maximal size that necessitates euthanasia. Tumour growth was markedly increased in FAM46C-depleted vs. Luciferase shRNA control MDA-MB-435 xenografts, beginning at approximately 2 weeks after subcutaneous injection (Figure 7.1a,b), supporting FAM46C’s putative role as a tumour suppressor and indicating potential regulation of Plk4 activity by FAM46C in vivo. Tumour weight at time of sacrifice was correspondingly increased at 3 weeks in FAM46C-depleted tumours (Figure 7.1c). Interestingly, while depletion of Plk4 or FAM46C with the respective shRNAs in xenografts was persistent over the course of the experiment, FAM46C shRNA in this system resulted in a modest increase in Plk4 mRNA expression (Figure 7.1d). Increased FAM46C expression results in reduced Plk4 autophosphorylation/activity, while FAM46C depletion results in centriole overduplication, indicating regulation at the protein level. It is also possible that FAM46C regulates Plk4 at the level of mRNA expression. Xenografts with co-depletion of both Plk4 and FAM46C showed an intermediate growth profile and tumour weight at sacrifice when compared to Luciferase+Plk4 shRNA and Luciferase+FAM46C shRNA xenografts (Figure 7.1e,f). Future work can focus on expanding the preliminary data we have obtained on the role of FAM46C in regulating Plk4. To identify the mechanism of FAM46C’s action on Plk4, Plk4 and FAM46C deletion mutants can be used in co-immunoprecipitation assays to determine which domains are interacting, and crystallization studies can be utilized for structural analysis. Crystallizing FAM46C alone can identify its molecular structure, providing
insight on how its domains organize to interact with and autoregulate Plk4. Then co-crystallization with Plk4 can visualize how FAM46C is inhibiting Plk4 activity, whether this be by inhibiting dimer formation or some other mechanism. In vitro kinase assays with purified protein can also be utilized to determine the kinetics of the inhibition of Plk4 kinase activity by Plk4. Furthermore, if our group is able to obtain anti-pS305 Plk4 antibody, which labels phosphorylated active Plk4, we can show in immunofluorescence assays whether FAM46C overexpression or depletion is affecting Plk4 activity as well as abundance at the centriole. Moreover, as FAM46C is a conserved protein, we can evaluate whether it functions similarly in another system, such as Drosophila. We can evaluate for physical interaction with co-immunoprecipitation assays and colocalization with SAK in Drosophila S2 cells. We can also see if its overexpression or depletion results in the same centriolar phenotypes observed in mammalian cells. In future experiments the effect of FAM46C on cell movement should be studied. It may be analogous to CEP192, which has been shown to be required for microtubule nucleation, efficient polarization and cell migration (O'Rourke, Gomez-Ferreria et al. 2014).
Figure 7.1

(a) Comparison of tumor appearance at 2 weeks and 3 weeks for Luciferase shRNA, Plk4 shRNA, and FAM46C shRNA.

(b) Graph showing tumor volume (mm³) over time (weeks) for Luciferase shRNA (n=17), Plk4 shRNA (n=18), FAM46C shRNA#1 (n=8), and FAM46C shRNA#2 (n=8).

(c) Bar graph comparing tumor weight (g) between different shRNA treatments.

(d) Scatter plots showing relative Plk4 mRNA expression (ΔΔCt) and relative FAM46C mRNA expression (ΔΔCt) for Luciferase shRNA, Plk4 shRNA, and FAM46C shRNA#1 and #2.

(e) Graphs showing tumor volume (mm³) and tumor weight (g) over time (weeks) for Luc+Plk4 shRNA (n=10), Luc+FAM46C shRNA (n=10), and Plk4+FAM46C shRNA (n=10).

(f) Scatter plots comparing relative Plk4 mRNA expression (ΔΔCt) and relative FAM46C mRNA expression (ΔΔCt) with Luciferase shRNA as the control.
Figure 7.1. Endogenous inhibitor of Plk4, FAM46C, modulates MDA-MB-435 xenograft tumour growth. a-c) MDA-MB-435 cells were injected into the right flanks of nude mice. a) Representative images of flank tumours in nude mice at the indicated times after injection. b) Tumour volume of xenografts at indicated times after subcutaneous injection, showing increased tumour size in FAM46C vs. Luciferase shRNA xenografts after 2 weeks, *p<0.05, **p<0.01 vs. Luciferase shRNA. c) Corresponding tumour weights after sacrifice, showing increased weight of FAM46C shRNA xenografts, *p<0.05 vs. Luciferase shRNA. d) Relative quantification of Plk4 and FAM46C mRNA levels in tumours at 3 weeks after injection, showing persistence of FAM46C or Plk4 depletion as appropriate, *p<0.001 vs. Luciferase shRNA. e) Tumour volume of xenografts at indicated times after subcutaneous injection, showing a partial rescue of the decreased tumour volume with Plk4 depletion in Plk4+FAM46C shRNA xenografts vs. Luciferase+Plk4 shRNA. Right panel, corresponding tumour weights showing an intermediate tumour weight in the Plk4+FAM46C shRNA xenografts vs. Luciferase+Plk4 shRNA and Luciferase+FAM46C shRNA tumours, *p<0.05 vs. Luciferase+Plk4 shRNA. f) Relative quantification of Plk4 and FAM46C mRNA levels in tumours at 3 weeks after injection, showing persistence of FAM46C or Plk4 depletion as appropriate. Top panel, *p<0.05 vs. Luciferase+FAM46C shRNA. Bottom panel, *p<0.05 vs. Luciferase+Plk4 shRNA. Data are means±SEM.
7.2.2. Determinants of sensitivity to targeted anti-Plk4 therapy.

Further efforts can also focus on characterizing a molecular profile that can predict success of anti-Plk4 therapy. While there was a clear rationale to develop selective Plk4 inhibitors for clinical use given Plk4’s importance for cell division, tumour growth, and its overexpression in common solid tumours (Macmillan, Hudson et al. 2001, Mason, Lin et al. 2014), there are several potential caveats regarding the clinical application of Plk4 inhibitor therapy. The highly selective Plk4 inhibitor, centrinone/centrinone B, is not orally bioavailable; its use in cancer patients in a parenteral form would require considerable preclinical testing. An oral formulation of this specific Plk4 inhibitor could potentially be developed and future efforts can focus on this (Wong, Anzola et al. 2015). The orally bioavailable agent, CFI-400945, which has been in Phase I testing for approximately 18 months (Mason, Lin et al. 2014, Sampson, Liu et al. 2015) inhibits both Plk4 and the mitotic checkpoint kinase Aurora B (Mason, Lin et al. 2014). The antitumour activity of CFI-400945 may thus be attributable in part to its Aurora B inhibition, so that patient selection based on a high level of tumour Plk4 expression may be unhelpful. This would also be true if the status of key Plk4 interactors in the individual patient tumour abrogated sensitivity to Plk4 inhibition. Future work can therefore focus on determining the key molecular interactors to be assessed in an individual patient’s cancer prior to selection of Plk4 inhibitor therapy. One approach can be to determine the sensitivity of human cancer cell lines to Plk4 inhibition based on Plk4 interactor status (Cep192, FAM46C, Ect2, ARHGEF1, Arp2, p53 and PTEN), with respect to Plk4’s known functions in regulating centriole number, proliferation, spreading, migration or invasion. This strategy is illustrated by the work Mason et al. have carried out to investigate the dependence of Plk4 inhibition on PTEN status: in particular these authors found that loss of PTEN increased sensitivity of cancer cell lines and xenografts to CFI-
In a more comprehensive look at PTEN and other interactors, shRNA or CRISPR-Cas can be used to deplete or overexpress these, in combination with Plk4 depletion/inhibition. These findings can then inform further studies utilizing the xenograft models described in this thesis, with evaluation of tumour growth, local invasion and metastasis formation upon altering expression of Plk4 and a candidate interactor. Our group has also established a 3D spheroid invasion model, where cells are plated on ultra low attachment plates to allow formation of spheres in culture and then embedded in Matrigel, which allows for cells to invade out of the spheres into the surrounding matrix. Using this model, we have also been able to show that Plk4 depletion suppresses 3D invasion in HeLa cells (Figure 7.2a). This assay can be utilized prior to the xenograft studies to evaluate invasion in a 3D system with combined alteration of Plk4 and interactor expression.
Figure 7.2

Luciferase shRNA

Plk4 shRNA#1

Plk4 shRNA#2
Figure 7.2. Plk4 promotes invasion in a 3D spheroid invasion assay. a) Representative brightfield images of HeLa cells plated on ultra low attachment plates, which form spheres in culture over 4 days. Addition of Matrigel stimulates outward invasion of cells from Luciferase shRNA spheroids, while invasion is suppressed in Plk4 shRNA spheroids, n=3 independent experiments.
7.2.3. Effect of Plk4 inhibition on immune cell function.

Another avenue of exploration is to assess the effect of Plk4 depletion/inhibition on non-cancer cells. The studies presented in this thesis have shown dependence of cancer cell migration and invasion on Plk4 but its depletion suppresses motility in normal cells (MEFs, HEK293T) as well (Rosario, Kazazian et al. 2015), suggesting potential for compromise of immune cell function and wound healing. Immune cell functional assays can be utilized to explore the effect of Plk4 depletion/overexpression/inhibition on immune cell function, including assessing neutrophil, macrophage or lymphocyte proliferation, migration wound healing, transwell migration/invasion and activity. Further studies can also assess wound healing \textit{in vivo} in mice treated with a Plk4 inhibitor, as well as using ELISpot assays (or ELISA and flow cytometry) to measure the cellular functions of immune system cells derived from these mice. Of note, Plk4 was originally described as highly expressed in lymphocytes (Fode, Binkert et al. 1996), and such studies could reveal a novel role for Plk4 in these cells.

7.2.4. Regulation of RhoGTPases by Plk4.

Plk4 has been shown to regulate the level of activation of the three main small RhoGTPases, RhoA, Rac1 and Cdc42 (this study, (Rosario, Ko et al. 2010, Brashavitskaya, Kazazian et al. 2013)); however, the mechanisms through which Plk4 activates these RhoGTPases, and what effect this has on cell motility, remain largely unknown. Plk4 has been shown to phosphorylate the RhoA GEF Ect2, which is required for cytokinesis. Our group showed that Ect2 is important for activation of RhoA by Plk4 in late mitosis (Rosario, Ko et al. 2010). Ect2 is considered an oncogene and is upregulated in human tumours including brain, lung, bladder, gastric, pancreatic, and ovarian cancer (Saito, Liu et al. 2004, Sano, Genkai et al. 2006, Salhia, Tran et
Moreover, it can localize to cell protrusions, in addition to the spindle midbody (Rosario, Kazazian et al. 2015), indicating a potential role in Plk4-related carcinogenesis or tumour progression, which can be explored in future studies. Our group has also interrogated a library of 145 GEFs, GAPs and GDI s for the core consensus phosphorylation motif for Plk4 (Leung, Ho et al. 2007, Sillibourne, Tack et al. 2010). By this strategy, 12 further potential substrates for Plk4 phosphorylation have been identified (ARHGEF1, PREX2, ALS2, PLEKHG6, RGNEF, TRIO, DOCK2, DOCK3 and DOCK5–8). Future studies can investigate these GEFs for activation of their predicted RhoGTPases in cancer cells, functions in cell motility/invasion, localization to protrusions, and functional interaction with Plk4 in promoting cell motility/invasion.

7.2.5. Plk4 and cell polarity.

Another potential area of investigation is to further explore the role of Plk4 in cell polarity. We show that Plk4 depletion alters reorientation of the Golgi/MTOC relative to the nucleus when cells are stimulated to migrate, and that correspondingly, Plk4 depletion results in less active Cdc42 in cancer cells. Polarization in general is regulated by an extensive intracellular signaling network, with correct polarization frequently lost in cancer. Conserved regulators of cell polarity, such as the Par complex, are shown to be important in tumourigenesis and polarized cell migration suggesting a role in malignant progression (Wodarz and Nathke 2007, Etienne-Manneville 2008). Cdc42 participates in polarized cell migration by regulating Par6 and aPKC (Anderson, Gill et al. 2008), as well as controlling actin polymerization and MTOC reorientation through other mechanisms (Cau and Hall 2005). The Par complex members Par6 and Par3 have
been shown to modulate RhoA and Rac1 activity (Wang, Zhang et al. 2003, Zhang and Macara 2006). The Par complex is also thought to function in EMT, with Par6-mediated degradation of RhoA shown to be necessary for tight junction dissolution and progression of the EMT pathway (Ozdamar, Bose et al. 2005). Par6 binding and phosphorylation by TGF-β, which has also shown to downregulate Par3 expression, induces translocation of Par6 from tight junctions to the cytoplasm (Wang, Nie et al. 2008), suggesting that TGF-B-induced EMT can be mediated by remodeling of the Par complex. Overall, dysregulation of normal polarity and the main components of the molecular machinery that create and maintain polarity and regulate migration are implicated in tumour progression, and further studies can elucidate the role of Plk4 in these pathways, for example by determining its effect on the localization or activation of the Par complex.

There are thus several avenues to explore in future studies that can help elucidate the specific pathways involved in Plk4-mediated tumour progression, some of which I have mentioned above. The findings of the Phase I clinical trial on human patients with advanced tumours utilizing the Plk4/AURKB inhibitor, CFI-400945, may yield initial information about conditional tumour sensitivity to Plk4/AURKB inhibition, though this was not the endpoint of the trial. Once safety is demonstrated in the Phase I component, Phase II trials can be designed to determine whether the inhibitor has any biological activity or effect. The preclinical studies described in this thesis should help inform this clinical experience, as our work has revealed a key molecular Plk4 interactor that can be assessed in a patient’s cancer prior to selection of Plk4 inhibitor therapy. Further exploration of the synergistic interaction between Plk4 and Arp2 would reveal which vulnerabilities are relevant in targeting tumour progression. It is possible that patients with
high Arp2 expression in their tumours may not benefit from Plk4 inhibition, and Plk4 expression level alone is not likely to be a reliable predictor of sensitivity to its inhibition. Moreover, an understanding of the role Plk4 is playing in a given individual tumour, in particular its relative contributions to growth- and motility-related cellular functions, will be important in appropriate selection of patients and in determining outcome measures for Phase II or III trials. Proliferation or local tumour growth alone may not be the most significant phenotype in disease progression, as seen in these studies where the effect of Plk4 on invasion and metastasis was the dominant factor, and outcome measures focusing on tumour size may not capture the efficacy of inhibitor treatment.
References


Hedley, D. (personal communication).


defects, and apoptosis."


regulated by PLK4 and targets HsSAS-6 to control centrosome duplication." Nat Cell Biol 13(8): 1004-1009.


Su, Y. C., J. Han, S. Xu, M. Cobb and E. Y. Skolnik (1997). "NIK is a new Ste20-related kinase that binds NCK and MEKK1 and activates the SAPK/JNK cascade via a conserved regulatory domain." EMBO J 16(6): 1279-1290.


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