The Role of Tiam1/Rac Signalling
in the Centriole Cycle

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<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>Anaphase promoting complex</td>
</tr>
<tr>
<td>AU</td>
<td>Absorbance unit</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Cas9</td>
<td>CRISPR-associated protein-9</td>
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<tr>
<td>CDC42</td>
<td>Cell division cycle 42</td>
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<tr>
<td>CDK1</td>
<td>Cyclin dependent kinase 1</td>
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<td>DAPI</td>
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<td>Dbl</td>
<td>Diffuse B-cell lymphoma</td>
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<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
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<td>Deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
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<tr>
<td>DOX</td>
<td>Doxycycline</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>Polyvinylidene fluoride</td>
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<tr>
<td>RBD</td>
<td>Ras binding domain</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>Rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>rtTA</td>
<td>Reverse tetracycline-controlled transactivator protein</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>S</td>
<td>S-phase</td>
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<tr>
<td>SAC</td>
<td>Spindle assembly checkpoint</td>
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<tr>
<td>SAS-4</td>
<td>Spindle assembly abnormal protein 4</td>
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<td>SAS-5</td>
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<td>SCF</td>
<td>Skp1–Cullin1–F-box protein</td>
</tr>
<tr>
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<td>Single guide ribonucleic acid</td>
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<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
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<tr>
<td>S-M linker</td>
<td>S-phase – mitosis linker</td>
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<td>Spindle defective 2</td>
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<tr>
<td>STIL</td>
<td>SCL/TAL1 interrupting locus</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>Tiam1</td>
<td>T-cell lymphoma invasion and metastasis 1</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet light</td>
</tr>
<tr>
<td>VSVG</td>
<td>Vesicular stomatitis virus glycoprotein</td>
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<tr>
<td>v/v</td>
<td>volume/volume</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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<tr>
<td>w/v</td>
<td>weight/volume</td>
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<tr>
<td>ZYG1</td>
<td>Zygotic defective: embryonic lethal 1</td>
</tr>
<tr>
<td>βTrCP</td>
<td>Beta-transducin repeat-containing protein</td>
</tr>
<tr>
<td>γTuRC</td>
<td>Gamma tubulin ring complex</td>
</tr>
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</table>
Abstract

The University of Manchester, Erinn-Lee Ogg
Degree: Doctor of Philosophy/PhD
Thesis title: The Role of Tiam1/Rac Signalling in the Centriole Cycle
Date: 2015

The mitotic spindle is a structure that facilitates the equal segregation of DNA in to two daughter cells during mitosis. The centrosomes organise the mitotic spindle in to a bipolar conformation, and this arrangement is essential for maintaining faithful chromosome segregation. Deviations in centrosome number alter the structure of the spindle and this can promote multipolar cell divisions and large scale aneuploidy. Therefore centrosome number is tightly regulated in normal cells to prevent interference with bipolar spindle assembly and faithful chromosome segregation.

Tumour cells often have supernumerary centrosomes. To prevent interference with bipolar spindle assembly extra centrosomes are clustered in to a bipolar spindle arrangement – preventing a multipolar cell division and intolerable levels of aneuploidy. However centrosome clustering can promote lagging chromosomes during anaphase, a major contributor to chromosomal instability. Chromosomal instability has been shown to enhance the malignant potential of tumour cells. Therefore it is important to understand the mechanisms involved in regulating centrosome number and how these become deregulated in cancer.

Centrosome number is regulated by the centrioles, small barrel shaped organelles found within the core of the centrosome. We have identified Tiam1, a Rac specific guanine nucleotide exchange factor, as a novel regulator the centriole cycle. Tiam1 knockdown is sufficient to promote the assembly of multiple aberrant centriole structures in human tumour cell lines. These aberrant centriole structures behave like functional centrioles and correlate with an increase in lagging chromosomes at anaphase – suggesting these structures are capable of promoting chromosomal instability in human tumour cell lines. Moreover we have shown that Tiam1 regulates centriole number by preventing centriole re-duplication events and significantly this effect is independent of Rac activation. We have also identified MCM5 as a novel Tiam1 interactor. MCM5 has been identified in the literature as a regulator of centrosome re-duplication. Therefore we propose that Tiam1 and MCM5 co-operate to prevent centriole re-duplication in human tumour cell lines. These findings identify Tiam1 as a potential prognostic marker of centriole amplification and chromosomal instability in human tumours.
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Chapter 1. Introduction

1.1 The genetic basis of cancer

Cancer is a complex genetic disease. Over 100 different cancer types have been identified in humans - and the genetic blueprint underlying each one of these cancers is distinct (Hanahan and Weinberg, 2000). Irrespective of the combination of genetic alterations within a cancer cell the phenotypic output is the same – a malignant cell is unresponsive to the normal stimuli that control a cell’s lifespan. Malignant characteristics can be generalised by the ‘hallmarks of cancer’ (Hanahan and Weinberg, 2011). Malignant cells have indefinite proliferative potential, they are unresponsive to cell death signals and can support their own growth and replication (Hanahan and Weinberg, 2011). They can also initiate angiogenesis as this is required to support the clonal expansion of malignant cells to form a tumour (Hanahan and Weinberg, 2011). Lastly malignant cells acquire characteristics which allow them to invade surrounding tissues and metastasise to additional sites within the body (Hanahan and Weinberg, 2011). Although the characteristics shared between malignant cells are the same the underlying defects which allow the development of these phenotypes are infinite.

1.1.1 Causes of genetic alterations in cancer

Malignant transformation begins with a mutation within a driver gene. This can result in the over-expression of a protein that promotes the enhanced survival of that cell (oncogene) - or this can result in the loss of a protein that limits cell growth and survival (tumour suppressor) (Boland and Ricciardiello, 1999). This mutational event can arise randomly, be inherited or be due to exposure to carcinogens. From this single mutational event, a cascade of events ensues which promotes the accumulation of multiple passenger mutations that enhance the survival of the malignant cell and its progeny (Nowell, 1976).

Aneuploidy is a common phenotype within tumour cells (Weaver and Cleveland, 2006). This is where a cell has an incorrect karyotype – the number of chromosomes deviates from normal. Therefore aneuploidy facilitates large scale genomic changes within the cell that has obvious consequences for gene expression.
The outcome of aneuploidy depends on the combination and number of chromosomes involved – aneuploidy has been linked to enhanced cell survival and decreased cell viability (Lynch et al., 1993; Weaver et al., 2007). Therefore the role of aneuploidy in tumourigenesis remains complex (Weaver and Cleveland, 2006; Torres et al., 2008). Aneuploidy is caused by defects during mitosis – resulting in the unequal segregation of DNA between daughter cells. In normal, untransformed cells mitosis is highly regulated to prevent chromosome segregation errors and potentially detrimental deviations in chromosome number.

1.2 The mitotic spindle

The mitotic spindle is a structure that facilitates the equal segregation of DNA in to two daughter cells during mitosis. Alterations in genomic content can have catastrophic effects on cell functioning and can have a negative effect on cell viability (Compton, 2010; Nasmyth, 2002). Therefore the equal segregation of DNA in to daughter cells is paramount for maintaining normal cell functioning.

The chromosomes are replicated during S-phase, producing two identical sister chromatids that need to be partitioned into two daughter cells. This is achieved by the mitotic spindle which promotes the bi-oriented association of microtubules (MTs) with kinetochores at the chromatids. The kinetochores found on a pair of sister chromatids are positioned in a back-to-back conformation which favours a bi-orientated MT attachment (Indjeian et al., 2007; Lončarek et al., 2007). A single kinetochore can associate with up to 25 MTs and it is crucial that each of these MTs are emanating from a single spindle pole, maintaining a bi-orientated relationship and faithful chromosome segregation (Maiato et al., 2004; Walczak and Heald, 2008). Therefore the conformation of the mitotic spindle is also crucial for maintaining faithful chromosome segregation (Blangy et al., 1995; Ganem et al., 2009). The centrosomes organise the structure of the mitotic spindle – when the cell enters mitosis the centrosomes are pushed apart to opposing sides of the cell producing a characteristic bipolar arrangement (Blangy et al., 1995; Tosó et al., 2009). Therefore the bipolarity of the spindle and the back-to-back conformation of the kinetochores ensures each sister chromatid is pulled towards opposing spindle poles, promoting the equal partitioning of DNA in to two daughter cells (Figure 1).
1.3 The centrosome

A pair of centrosomes form the poles of the mitotic spindle. The centrosome consists of a large network of proteins called the pericentriolar matrix (PCM) and embedded within this network is a pair of centrioles. Together they form the centrosome (Figure 2).

Figure 2. Schematic of the centrosome. The centrosome consists of a pair of centrioles (green) encased in PCM (grey).

The primary function of the centrosome is to nucleate MTs – therefore this organelle is regarded as the Microtubule Organising Centre (MTOC) of the cell. Using appendages arranged on the centriole, the centrosome can anchor itself to the plasma membrane resulting in localised regions of MT nucleation which protrude from the cell surface – producing cilia and flagella (Graser et al., 2007a; Ishikawa et al., 2005). Cilia and flagella are important for chemosensory and motility functions of the cell (Mitchell, 2007).
Cilia have also been identified as important signalling hubs, relaying information from the surrounding environment back to the cell (Goetz and Anderson, 2010). However when anchored to the cell membrane the centrosome is more commonly referred to as a basal body. The centrosome also plays a fundamental role in protein trafficking and regulation of cell shape and polarity (Azimzadeh and Bornens, 2007).

In mammalian cells 100 proteins have been associated with the PCM and the majority are large coiled-coil proteins (Anderson et al., 2003; Bettencourt-Dias and Glover, 2007; Jakobsen et al., 2011). The PCM serves as an anchoring and nucleation site for MTs (Azimzadeh and Bornens, 2007; Moritz et al., 2000). Therefore the PCM is responsible for the MT nucleating capacity of the centrosome. However the PCM is recruited by the centrioles, small barrel shaped organelles found embedded within the PCM (Basto et al., 2006; Bobinnec et al., 1998). Therefore the centrioles are an important component of the centrosome in mammalian cells.

### 1.4 The centriole

The centriole is a small barrel shaped organelle. At its proximal end a cartwheel structure forms the base of the centriole which templates its unique nine-fold symmetry (Nakazawa et al., 2007). Nine triplets of centriolar MTs connect to this cartwheel forming the walls of the barrel. The nine triplets are made up of A, B and C-tubules which are organised in an anti-clockwise arrangement (Paintrand et al., 1992; Rustem and Uzbekov, 2007). Each triplet is attached to the next by an A-C linker that connects A and C-tubules (Figure 3A) (Paintrand et al., 1992). The walls of the centriole elongate by extending the A and B-tubules – the length of each tubule is regulated by a delicate balance between MT nucleation factors and MT stabilisation factors (Gönczy, 2012; Paintrand et al., 1992; Schmidt et al., 2009). Once the barrel has reached its optimum length (~500nm) a capping protein sits on the distal end of the centriole preventing any further centriole elongation (Figure 3B) (Azimzadeh and Marshall, 2010; Gönczy, 2012). In addition, the centriolar MTs are heavily acetylated and polyglutamylated ensuring the centriolar MTs do not mirror the dynamic nature of the cytoplasmic MTs (Edde et al., 1990; Kochanski and Borisy, 1990; Piperno and Fuller, 1985). Together this ensures the centriole architecture is maintained over numerous generations. When the centriole is fully matured it acquires two sets of appendages on its distal end (Figure 3C) (Paintrand et al., 1992).
These appendages allow the centriole to attach itself to the plasma membrane (Azimzadeh and Marshall, 2010).

Figure 3. The structure of the centriole. A) The proximal base of the centriole resembles a cartwheel structure from which nine triplets of A, B, and C centriolar MTs radiate. B) The walls of the centriole consist of MT triplets which extend in to duplets at the distal end. The length of the centriole is regulated by a distal cap. C) The mature centriole is decorated with appendages on its distal end. These allow the centriole to anchor to the plasma membrane. (Azimzadeh and Marshall, 2010).

1.4.1 Overview of centriole biogenesis

The construction of this elaborate organelle is still not fully understood. Key experiments in the model system Caenorhabditis elegans (C. elegans) discovered that the sequential recruitment of five core centriolar proteins is essential for centriole biogenesis (Delattre et al., 2006; Gönczy, 2012; Pelletier, 2006). Spindle defective 2 (SPD-2), a large coiled coil protein is the first protein to be found localised at the newly assembled centriole in C. elegans embryos (Delattre et al., 2006). This triggers the recruitment of Zygotic defective: embryonic lethal 1 (ZYG-1) to the centriole (Delattre et al., 2006). Following recruitment of ZYG-1, a complex of an additional two coiled coil proteins, SAS-5 and SAS-6 is localised at the centriole (Delattre et al., 2006). This complex initiates the assembly of the central tube that forms the core of the new centriole barrel (Pelletier, 2006).
The SAS-5/SAS-6 complex also plays a role in mediating the length of the central tube (Pelletier, 2006). It has been shown that ZYG-1 is essential for the assembly of this central tube as it phosphorylates SAS-6, ensuring it remains localised at the assembling centriole (Kitagawa et al., 2009). Following assembly of the central tube, SAS-4 is recruited to the newly forming centriole (Delattre et al., 2006). SAS-4 is another coiled coil protein which assembles centriolar MTs around the outside of the central tube, forming a completed barrel shaped organelle (Pelletier, 2006). Notably homologues of each of these proteins have been identified in other systems including humans (Azimzadeh and Marshall, 2010; Gönczy, 2012; Hodges et al., 2010). Although this model identified in C. elegans dramatically simplifies the centriole assembly process, it has served as a crucial starting point to further elucidate the process in other systems.

1.4.2 Centriole biogenesis in humans

The five ‘core’ components identified in C. elegans have been identified within human cells. These proteins exhibit similar functional roles to those identified in C. elegans and they retain a step-wise manner of recruitment to the centrioles to facilitate centriole assembly. Polo kinase 4 (PLK4) mirrors the role of ZYG-1. PLK4 is regarded as the master regulator of centriole biogenesis and is required for the stabilisation of human SAS-6 at the centrioles (Habedanck et al., 2005; Puklowski et al., 2011). Human SAS-6 is required for the localisation of STIL and CPAP, resembling the relationship between SAS-6/SAS-5 and SAS-4 in C. elegans (Tang et al., 2011). However STIL and CPAP have been shown to interact directly in human cells, forming a complex with human SAS-6 (Tang et al., 2011). Loss of STIL prevents the localisation of human SAS-6 at the centrioles and CPAP recruitment is dependent on the localisation of human SAS-6 and STIL (Arquint et al., 2011; Tang et al., 2011). Therefore these three proteins co-operate to promote centriole biogenesis (Arquint et al., 2011; Tang et al., 2011). Taken together the sequential recruitment of proteins is also important for centriole biogenesis in human cells. From this basic scaffold initially identified in C. elegans, numerous additional proteins have been identified as important regulators of centriole biogenesis in humans.
1.4.2a Building the proximal end of the centriole

PLK4 is the master regulator of centriole biogenesis. This protein initiates the sequential recruitment of proteins required for centriole assembly. Loss of this protein is sufficient to prevent centriole biogenesis while over-expression of PLK4 causes excessive centriole assembly (Bettencourt-Dias et al., 2005; Kleylein-Sohn et al., 2007). Cep152 has been shown to interact with PLK4 (Hatch et al., 2010). Loss of Cep152 also prevents centriole biogenesis and prevents the downstream localisation of SAS-6 and CPAP to the centrioles (Cizmecioglu et al., 2010; Hatch et al., 2010). Therefore it is likely that Cep152 cooperates with PLK4 in the initial stages of centriole biogenesis to induce procentriole assembly. Cep63 has also been shown to interact with Cep152 and both proteins co-localise at the site of new centriole assembly (Sir et al., 2011). Loss of Cep63 prevents the localisation of Cep152 at the centriole, resulting in a reduction in centriole biogenesis (Sir et al., 2011). Therefore PLK4, Cep152 and Cep63 are collaboratively implicated in the early stages of centriole biogenesis. In C. elegans SAS-6 initiates the formation of a central tube which forms the core of the centriole barrel. However in humans the centriole base is formed from an intricate cartwheel structure (Gönczy, 2012; Paintrand et al., 1992). Human SAS-6 is responsible for the assembly of this cartwheel platform and is essential for maintaining the unique nine-fold symmetry of the centriole (Leidel et al., 2005; Nakazawa et al., 2007). The walls of the centriole barrel are assembled directly on to this platform (Gönczy, 2012).

SPD-2 was identified as an upstream signalling molecule required for the initial recruitment of ZYG-1 to the centrioles in C. elegans. Cep192 – the potential homolog of SPD-2 - is predominantly associated with the PCM and recruits proteins to the centrosome that are required for MT nucleation (Gomez-Ferreria et al., 2007). Therefore Cep192 is an essential component of the centrosome maturation machinery (Gomez-Ferreria et al., 2007). Loss of Cep192 results in mitotic arrest due to the assembly of an unstable spindle however the centrosomes in these cells retained centrioles (Gomez-Ferreria et al., 2007). Therefore the centriole biogenesis pathway is still initiated in the absence of Cep192 (Gomez-Ferreria et al., 2007). The PCM has been shown to facilitate centriole duplication and although loss of Cep192 does not prevent centriole assembly, it does result in a reduction in the number of centrioles assembled (Dammermann et al., 2004; Lončarek et al., 2008; Zhu et al., 2008). Therefore Cep192 is still implicated in the initial stages of centriole biogenesis pathway.
1.4.2b Building the walls of the centriole

The walls of the centriole consist of nine triplets of centriolar MTs (Gönczy, 2012; Paintrand et al., 1992). Gamma (γ) tubulin localises to the proximal base of the centriole where it forms a characteristic ring structure called the γ tubulin ring complexe (γTuRC) (Dammermann et al., 2004; Guichard et al., 2010). The γTuRC forms a scaffold which is necessary for the nucleation of centriolar MTs (Guichard et al., 2010). This scaffold facilitates the nucleation of the A-tubule which then supports the assembly of both the B- and C-tubules (Guichard et al., 2010). γ tubulin has also been shown to interact with CPAP (Hung et al., 2000). CPAP binds to α – β tubulin dimers, sequestering them in a non-polymerisable complex (Cormier et al., 2008). Although the full functional significance of this is unclear, this must be important for isolating a pool of tubulin to construct the centriole barrel – promoting centriole elongation (Cormier et al., 2009). Additional proteins have also been implicated in the regulation of centriole nucleation including POC1 (Keller et al., 2009), POC5 (Azimzadeh et al., 2009), Ofd1 (Singla et al., 2010) and Centrobin (Gudi et al., 2011).

As the centriole barrel elongates, protein complexes are assembled within the distal lumen of the organelle. These protein complexes assemble as elaborate stacks of tilted discs (Paintrand et al., 1992; Ibrahim et al., 2009) primarily composed of centrin – a calcium binding protein (Paoletti et al., 1996). These discs appear to be connected to the A-tubules of the barrel wall (Paintrand et al., 1992; Ibrahim et al., 2009). Therefore centrin is stably integrated within the core of the centriole organelle. Loss of centrin has been shown to prevent procentriole assembly in human cells (Salisbury et al., 2009). Therefore centrin is another important component of the centriole biogenesis pathway.

1.4.2c Building the distal end of the centriole

Procentriole length is controlled by a delicate balance between MT nucleation factors (CPAP etc) and MT stabilisation factors – this ensures every centriole reaches the same length (~500nm) (Schmidt et al., 2009). Cp110 has been identified as a MT stabilising factor, preventing excessive procentriole elongation.
Cp110 was found to localise to the ends of growing procentrioles early on within the biogenesis pathway (Kleylein-Sohn et al., 2007). Therefore it is believed that the centriolar MTs are inserted underneath this Cp110 cap (Kleylein-Sohn et al., 2007). Loss of Cp110 promotes the formation of excessively long procentrioles (Schmidt et al., 2009; Spektor et al., 2007). Therefore Cp110 is involved in stabilising the centriolar MTs and is considered to be essential for procentiole assembly.

The completed elongation of the centriole wall signifies the end of procentriole assembly. When the centriole fully matures - a process that takes roughly 1.5 cell cycles – appendages are assembled on the distal end of the centriole (Azimzadeh and Marshall, 2010). These appendages are essential for cilia formation and are made up of proteins such as Cep164 and OFD2 (Graser et al., 2007a; Ishikawa et al., 2005).

1.5 Centriolar satellites

Centriolar satellites are electron dense structures that have been found to localise in close proximity to the centrosome. These structures were first visualised next to assembling procentrioles (Bärenz et al., 2011). However these structures have now been shown to shuttle between the cytoplasm and the centrosome throughout the cell cycle (Bärenz et al., 2013; Kubo et al., 1999). These structures can interact with MTs and have been shown to localise to the centrosomes in a dynein dependent manner (Balczon et al., 1999; Kubo et al., 1999). PCM-1 is regarded as the main protein component of centriolar satellites (Kubo et al., 1999). Knockdown of this protein and therefore removal of centriolar satellites causes a reduction in the localisation of the following components at the centrosome; centrin, pericentrin and ninein (Dammerman and Merdes, 2002). It is thought that centriolar satellites can act as platforms within the cytoplasm that facilitate the modification of proteins that are required for centrosome functioning and in turn facilitate their transport to the centrosome (Dammerman and Merdes, 2002). Therefore the centriolar satellites are important for maintaining proper centrosome functioning. It has also been shown that excessive centriolar satellite formation precedes centrosome amplification in response to DNA damage (Löffler et al., 2013). Therefore centriolar satellites can facilitate the assembly of supernumerary centrosomes.
1.6 The centriole cycle

The centriole cycle follows a stepwise, cyclic pattern similar to the cell cycle. This ensures the centrioles are only duplicated once per cell cycle. Additionally centriole duplication is semi-conservative, similar to DNA replication. The resident centriole, termed the ‘mother’, templates the formation of a new daughter centriole - also known as the procentriole (Paintrand et al., 1992). The mother is permitted to produce a single daughter centriole per cell cycle (Kleylein-Sohn et al., 2007; Strnad et al., 2007). Taken together this ensures that the number of centrioles – and therefore centrosomes – found within a cell does not deviate from generation to generation.

1.6.1 G1 phase and the centriole cycle

Upon entry in to G1 the cell inherits a single centrosome containing two centrioles. The centriole pair is connected at their distal ends by a loose tether called the G1-G2 tether. This tether ensures the centrioles remain within microns of each other, allowing them to function collectively as a single centrosomal unit (Figure 4A) (Piel et al., 2000; Nigg and Stearns, 2011). Several proteins have been implicated in the functioning of the G1-G2 tether including CNap1, Rootletin, Cep68 and Cep215 (Bahe et al., 2005; Graser et al., 2007b; Mayor et al., 2000).

Centriole duplication is initiated during the G1-S transition by PLK4 (Habedanck et al., 2005). PLK4 is responsible for the stabilisation of SAS-6, the protein which is crucial for assembling the cartwheel structure at the proximal end of the newly formed procentriole (Gopalakrishnan et al., 2010; Nakazawa et al., 2007; Puklowski et al., 2011). PLK4 has been shown to stabilise SAS-6 indirectly via the ubiquitin ligase F-box and WD repeat domain containing 5 (FBXW5) (Puklowski et al., 2011). The levels of PLK4 and therefore SAS-6 are crucial for initiating the assembly of a new procentriole (Kleylein-Sohn et al., 2007; Puklowski et al., 2011). Over-expression of PLK4 alone is sufficient to promote centriole amplification (Kleylein-Sohn et al., 2007), whilst conversely, loss of PLK4 is sufficient to prevent centriole biogenesis (Bettencourt-Dias et al., 2005). Given this, the expression of PLK4 is regulated in a cyclic manner. PLK4 levels peak during mitosis and are subsequently down-regulated in the following interphase (Brownlee et al., 2011; Kleylein-Sohn et al., 2007; Rogers et al., 2009).
Therefore the temporal control of PLK4 is crucial for ensuring centriole duplication is initiated once per cell cycle. This identifies the first mechanism that limits centriole duplication to once per cell cycle.

Another protein which has been identified as an initiator of centriole duplication at the G1/S phase boundary is Cyclin dependent kinase 2 (CDK2) (Matsumoto et al., 1999). Classically CDK2 is known for its regulatory role in the cell cycle – CDK2 initiates the G1 to S phase transition. When CDK2 is complexed with Cyclin E this licenses the passage of the cell into S-Phase (Koff et al., 1992). The stepwise manner of the cell cycle is regulated by the temporal activation of CDKs by their corresponding Cyclins. For example the subsequent association of CDK2 which Cyclin A is important for the initiation of DNA replication (Pagano et al., 1992). Interestingly both CDK2-Cyclin E and CDK2-Cyclin A have been implicated in the initiation of centriole duplication (Meraldi et al., 1999; Okuda et al., 2000). Okuda and colleagues were able to demonstrate that nucleophosmin is a target of CDK2-Cyclin E. Nucleophosmin is localised at the centrioles and its dissociation is required for the initiation of centriole biogenesis. This dissociation event is dependent on the phosphorylation of nucleophosmin by CDK2-Cyclin E (Okuda et al., 2000). Furthermore CDK2-Cyclin A has been shown to phosphorylate the kinase MPS1 (Kasbek et al., 2007). MPS1 is localised at the centrosomes and its stabilisation (by phosphorylation) is required for centrosome duplication (Fisk and Winey, 2001; Fisk et al., 2003). Interestingly ectopic expression of CDK2 facilitates centriole amplification (Matsumoto et al., 1999). Therefore CDK2 couples centriole duplication with DNA replication – ensuring centriole duplication is only initiated once per cell cycle.

Therefore during G1 the centrioles are not in physical contact with each other but are connected by their proximal ends to ensure they function collectively as a single centrosomal unit. Furthermore there is the up-regulation and activation of proteins that are essential for centriole biogenesis. The temporal regulation of these proteins is crucial for ensuring centriole duplication is initiated once per cell cycle.
1.6.2 S-phase and the centriole cycle

Procentriole biogenesis is initiated during the G1-S transition by the up-regulation of proteins required for the initiation of centriole duplication. As discussed in Section 1.4.2 the activation of PLK4 initiates the sequential recruitment of centriolar components required for the assembly of the centriole organelle (Habedanck et al., 2005; Kleylein-Sohn et al., 2007). PLK4 levels are subsequently down-regulated during S-phase to prevent the initiation of additional duplication events. PLK4 regulates its own expression in a negative feedback loop. The autophosphorylation of PLK4 is recognised by the ubiquitin ligase Skp1 – Cullin 1 – F-box Beta-transducin repeat-containing protein (SCFβTrCP), resulting in the proteosomal degradation of PLK4 (Cunha-Ferreira et al., 2009; Guderian et al., 2010; Holland et al., 2010). The precise regulation of PLK4 levels permits each centriole to produce a single daughter procentriole, yielding a total of four centrioles per cell.

The newly formed procentriole is assembled directly on to the lateral side of the mother (Paintrand et al., 1992). This gives the centrioles a characteristic ‘L shape’ during S-phase (Figure 4B). When the mother and daughter centriole are in this conformation they are described as ‘engaged’. This method of assembly is believed to be an additional regulatory mechanism to ensure that centriole duplication is initiated once per cell cycle. The centrioles are held in this characteristic ‘L shape’ by a connection called the S-M linker. Little is known about the protein composition of the S-M linker. Studies in drosophila have shown that SAS-6 and Ana2 are involved in its functioning (Stevens et al., 2010). It has been shown that the establishment of the S-M linker is sufficient to suppress additional duplication events. Engaged centrioles are unable to replicate when put in an environment that supports centriole biogenesis – i.e. S-phase (Wong and Stearns, 2003). However when the S-M linker is removed, the centrioles can over-duplicate in S-phase (Lončarek et al., 2008). This supports the idea that the physical connection between the centrioles can provide an additional block to duplication. Given this data it has been hypothesized that the establishment and maintenance of the S-M linker is another regulatory mechanism ensuring centriole duplication occurs once per cell cycle. Exactly how this linker prevents additional rounds of duplication is unknown. It was suggested that there is a single site on the lateral side of the mother centriole that is capable of assembling a daughter procentriole. Therefore keeping the newly formed procentriole attached to the mother simply blocks the single available site of centriole biogenesis.
Although this is an attractive model, studies with PLK4 have shown that over-expression of this protein causes the assembly of multiple daughter centrioles around the entirety of the barrel of the mother (Kleylein-Sohn et al., 2007). Therefore procentrioles can be forced to assemble at multiple sites on the mother. Notably this occurs when the S-M linker is still intact. Taken together, the exact functional role of the S-M linker remains unclear.

Therefore during S-phase the centrioles duplicate semi-conservatively with the procentriole being assembled directly on the lateral side of the mother. Following the initiation of procentriole assembly it is crucial to negatively regulate the proteins that are involved in initiating centriole biogenesis – this prevents the assembly of multiple centrioles. Furthermore the maintenance of the S-M linker prevents the initiation of additional duplication events. Taken together it is clear that the stepwise manner of the centriole cycle relies heavily on the timed expression and/or availability of proteins that are required for centriole biogenesis.

1.6.3 G2 phase and the centriole cycle

The newly synthesised procentriole is considerably shorter than the mother. During G2 the procentriole elongates to approximately 500nm in length (Figure 4C) (Azimzadeh and Marshall, 2010; Schmidt et al., 2009). The newly formed centriole pairs begin to prepare for the onset of mitosis. Mitosis requires the co-operated functioning of two independent centrosomes - each containing two engaged centrioles. There is an up-regulation of Aurora A and Polo kinase 1 (PLK1) which facilitates the recruitment of additional PCM components (Berdnik and Knoblich, 2002; Hannak et al., 2001). This is in preparation for the increased nucleation capacity required by the centrosomes during mitosis.

Each engaged centriole pair with a full complement of PCM needs to function as an independent centrosome. However the original resident centrioles are attached at their distal ends by the G1-G2 tether. This tether must be disassembled during G2 to allow centrosome separation which is fundamental for bipolar spindle assembly during mitosis (Figure 4C). Disassembly of the G1-G2 tether is achieved by a kinase called NEK2. NEK2 belongs to the Never In Mitosis A (NIMA) family of protein kinases (Faragher and Fry, 2003).
NEK2 only becomes active when its expression levels exceed the levels of its counteracting phosphatase – type 1 gamma phosphatase (Helps et al., 2000). Active NEK2 has been shown to phosphorylate CNap1 and rootletin, two protein components of the G1-G2 tether, resulting in its breakdown (Bahe et al., 2005; Fry et al., 1998).

Therefore the main focus of G2 and the centriole cycle is to ensure that the newly formed centriole pairs are prepared for the onset of mitosis. This requires the procentriole to reach maximum length and a surplus of PCM to be recruited to each centriole pair. This ensures each centriole pair is associated with its own cloud of PCM – without PCM the centrioles would be unable to nucleate MTs (Azimzadeh and Bornens, 2007; Moritz et al., 2000). Furthermore another crucial step is initiated during G2, the G1-G2 tether is broken down. This allows the centrosomes to function as two independent units in preparation of mitosis. Each of these key features is controlled by the timed expression of regulatory proteins, further highlighting the importance of the temporal regulation of proteins involved in the centriole cycle.

1.6.4 Mitosis and the centriole cycle

During mitosis the centrosomes facilitate the assembly of the mitotic spindle and centrosome separation is a fundamental step in this process. Kinesin 5 (Eg5) is a plus-end directed MT motor protein that localises specifically to the spindle MTs (Sawin and Mitchison, 1995). Eg5 facilitates bipolar spindle assembly by sliding anti-parallel spindle MTs apart – promoting centrosome separation (Kapitein et al., 2005). Negative forces are generated by dynein, CLIP 170, Lis1 and Tiam1 (Tanenbaum et al., 2008; Woodcock et al., 2010). These proteins counteract the movement generated by Eg5. This ensures the centrosomes do not migrate too far as this would also disrupt bipolar spindle assembly (Tanenbaum et al., 2008; Woodcock et al., 2010). Therefore the centrosomes play a fundamental role in mitosis – promoting the assembly of the bipolar spindle which is essential for faithful chromosome segregation.

During mitosis the centrioles are primed for another round of duplication in the subsequent cell cycle. The S-M linker that holds the centriole pairs in an engaged conformation must be removed to license further procentriole biogenesis (Figure 4D).
Degradation of this linker – or ‘disengagement’ of the centrioles – is controlled by Polo-like kinase 1 (PLK1) and separase (Tsou et al., 2009). Significantly these two proteins are predominantly active in mitosis. PLK1 is responsible for the activation of the Anaphase Promoting Complex (APC) (Hansen et al., 2004). The APC is activated when all the chromatids are successfully attached to MTs – ensuring the chromatids are faithfully segregated (Nasmyth, 2002). Separase is responsible for the degradation of cohesion complexes that hold sister chromatids together (Hauf et al., 2001). Breakdown of cohesion is necessary for chromosome segregation during anaphase. The activity of separase relies on the degradation of securin – a process that is initiated by the activation of APC (Hagting et al., 2002). Co-ordinating the breakdown of the S-M linker with anaphase onset and sister chromatid separation prevents licensing of centriole duplication prior to anaphase – avoiding promiscuous duplication events that may interfere with bipolar spindle assembly.

In conjunction with centriole disengagement, PLK4 must be up-regulated in preparation for centriole duplication in the next cell cycle. Twins, the regulatory subunit of protein phosphatase 2A (PP2A) is up-regulated during mitosis (Brownlee et al., 2011). This protein de-phosphorylates PLK4, providing a short window of PLK4 activity which is sufficient to promote centriole duplication in the subsequent cell cycle (Brownlee et al., 2011).

Therefore during mitosis the centrosomes are fundamental for facilitating faithful chromosome segregation. However the life cycle of the centrioles also relies on proteins specifically expressed during mitosis. Disengagement of the centrioles during anaphase presents a key licensing step for permitting centriole duplication in the subsequent cell cycle. Additionally the coordination of disengagement with anaphase ensures promiscuous duplication events cannot occur prior to anaphase – preventing any potential interference with bipolar spindle assembly and faithful chromosome segregation. Again this highlights that the temporal expression of proteins throughout the centriole cycle is fundamental for limiting centriole duplication to once per cell cycle.
Figure 4. The centriole cycle. A) During G1 the centrioles are disengaged but remain attached by a tether connecting their proximal ends (the G1-G2 tether). B) The centrioles duplicate semi-conservatively during S-phase with each mother centriole producing a single daughter procentriole (light green). C) During the early stages of G2 the daughter centriole elongates to reach the length of the mother. As the cell prepares to enter mitosis the centriole pairs recruit additional PCM and the G1-G2 tether is broken down. D) During mitosis each engaged centriole pair functions as an independent centrosome. Following completion of chromosome segregation centriole disengagement is initiated – licensing centriole duplication. Figure adapted from Nigg and Stearns, 2011.
1.7 Deviations in centrosome number interfere with bipolar spindle assembly

It is clear that the centriole cycle is highly regulated to prevent deviations centrosome number. This is because the centrosomes control the formation of the mitotic spindle and the bipolarity of the spindle is essential for maintaining faithful chromosome segregation. Although it has been shown that mitosis can be completed successfully without a spindle (Basto et al., 2006), interfering with the bipolarity of the spindle when present can have catastrophic effects on cell viability. Alterations to the spindle structure will cause large scale aneuploidy and ultimately cell death in normal, untransformed cells (Ganem et al., 2009).

Disruption of the bipolarity of the spindle can occur via errors in centrosome positioning. Failure of two centrosomes to separate to opposing sides of the cell at the onset of mitosis will cause a monopolar spindle formation, preventing chromosome segregation (Blangy et al., 1995). Additionally, errors in centrosome number will result in aberrant spindle morphologies. Having an excess of two centrosomes will result in the formation of a multipolar spindle. Cytokinesis usually fails in cells which attempt to complete a multipolar division, resulting in bi/poly-nucleated progeny and cell cycle arrest (Ganem et al., 2009). However, cells which do successfully complete mitosis with a multipolar spindle will segregate their DNA between too many daughter cells, resulting in progeny which are highly aneuploid and ultimately inviable (Ganem et al., 2009). Therefore in terms of normal, untransformed cells, having an excessive number of centrosomes is extremely detrimental. For this reason the centriole cycle is a highly regulated process to ensure each cell maintains two centrosomes.

1.8 Centrosome amplification and tumour cells

It is clear that there is extensive effort to prevent deviations in centriole number in normal, untransformed cells and centrosome amplification is rarely observed in normal human tissue (Nigg, 2002; Pihan et al., 1998; Sato et al., 1999). However a significant proportion of solid tumours show increased centrosome number (Kawamura et al., 2004; Landen et al., 2007; Lingle and Salisbury, 2000; Mayer et al., 2003; Nakajima et al., 2004; Nigg, 2002; Pihan et al., 1998; Pihan et al., 2003; Reiter et al., 2009; Sato et al., 1999; Skyldberg et al., 2001).
Therefore centrosome amplification must provide a beneficial phenotype to human tumour cells, particularly as supernumerary centrosomes are apparent in such a high proportion of tumours from different tissues of origin. Initially it was thought that tumour cells benefit from having supernumerary centrosomes because they can cause multipolar spindle formation, promoting aneuploidy (Ganem et al., 2009). However mitosis is rarely successful using a multipolar spindle (Ganem et al., 2009). If progeny are produced from a multipolar division the daughter cells cannot propagate (Ganem et al., 2009). Therefore it is thought that tumour cells can tolerate having too many centrosomes in two ways – by inactivating additional centrosomes or clustering additional centrosomes (Basto et al., 2008; Quintyne et al., 2005). When supernumerary centrosomes are inactivated they do not exhibit any association with MTs and become detached from the spindle poles during mitosis (Basto et al., 2008). This ensures additional centrosomes cannot interfere with bipolar spindle assembly. However if this is the principle mechanism allowing tumour cells to tolerate supernumerary centrosomes, this does not explain why such a high proportion of tumour cells maintain an excessive number of centrosomes – this would not provide the tumour cell with a selective advantage. Therefore centrosome clustering may be the principle mechanism adopted by tumour cells to tolerate supernumerary centrosomes (Godinho and Pellman, 2014; Quintyne et al., 2005). When the tumour cell enters mitosis, its supernumerary centrosomes are scattered throughout the cell. To maintain a bipolar spindle arrangement the centrosomes are pulled together by a combination of MT associated motor proteins (Karsenti and Vernos, 2001; Kwon et al., 2008). This allows mitosis to complete successfully without forcing a multipolar cell division – preventing intolerable levels of aneuploidy (Ganem et al., 2009). Superficially centrosome clustering appears to maintain faithful chromosome segregation due to the maintenance of a bipolar spindle. However it has been shown that clustering of supernumerary centrosomes is a prominent cause of chromosome mis-segregation (Ganem et al., 2009; Silkworth et al., 2009).

During the clustering process several transient multipolar intermediates are generated and these intermediates can nucleate MTs towards the chromatids. This provides a small window of opportunity for the tumour cell to promote inappropriate MT-kinetochore attachments at the chromatids (Figure 5) (Ganem et al., 2009; Silkworth et al., 2009).
1.8.1 Centrosome clustering and inappropriate MT-kinetochore attachments

As discussed in Section 1.2, the bipolarity of the spindle favours bi-orientated MT-kinetochore attachments at the chromatids. These are commonly referred to as amphitellic MT-kinetochore attachments and these attachments promote faithful chromosome segregation (Figure 6A) (Thompson and Compton, 2008). Centrosome clustering can promote the following inappropriate MT-kinetochore attachments at the chromatids:

1.8.1a Monotelic attachment

A monotelic attachment is when a kinetochore in a pair of sister chromatids fails to make contact with a MT (Thompson et al., 2010). When anaphase is licensed, the cohesion complex holding identical sister chromatids together is cleaved (Hauf et al., 2001; Nasmyth, 2002). This permits the segregation of identical DNA between two daughter cells. However if one sister chromatid fails to make contact with a MT - following degradation of cohesion - this chromatid will not be segregated in to any daughter cell. This will result in an aneuploid daughter cell (Figure 6B).
1.8.1b Syntelic attachment

Each kinetochore belonging to a pair of sister chromatids should be attached to MTs radiating from different spindle poles. However, in the case of a syntelic attachment, both kinetochores are attached to MTs radiating from the same spindle pole (Thompson et al., 2010). Following completion of anaphase this will result in two copies of the same chromosome residing within the same daughter cell - promoting aneuploidy (Figure 6C).

1.8.1c Merotelic attachment

A merotelic attachment occurs when a single kinetochore is attached to MTs radiating from both spindle poles (Thompson et al., 2010). When chromosome segregation is licensed the chromatid held by a merotelic attachment is pulled towards both spindle poles – promoting a ‘lagging chromosome’. The destination of this chromatid is completely dependent on which spindle pole lets go first, potentially segregating the chromatid in to the wrong daughter cell (Figure 6D). Merotelic attachments have also been shown to cause chromosome breaks, resulting in chromosome translocations (Janssen et al., 2011). Taken together a merotelic attachment has the potential to promote aneuploidy in daughter cells.
Figure 6. MT-kinetochore attachments. A) Amphitelic attachment. Each kinetochore is attached to separate spindle poles, promoting equal chromosome segregation. B) Monotelic attachment. A kinetochore remains unattached to a spindle pole. During chromosome segregation this chromatid will not be segregated in to a daughter cell – promoting aneuploidy. C) Syntelic attachment. Each kinetochore is attached to the same spindle pole. Following chromosome segregation both chromatids will reside in the same cell – promoting aneuploidy. D) Merotelic attachment. A kinetochore is attached to MTs from both spindle poles. This destination of this chromatid following chromosome segregation is down to which MT detaches first – potentially promoting aneuploidy. Figure adapted from Thompson et al., 2010.
1.9 Repair of inappropriate MT-kinetochore attachments

The spindle assembly checkpoint (SAC) plays a key role in resolving inappropriate MT-kinetochore attachments back to an amphitelic conformation. The SAC can identify unattached kinetochores (the product of a monotelic attachment) (Nezi and Musacchio, 2009; Reider, 1995; Thompson et al., 2010) and a lack of tension across the spindle (result of a syntelic attachment) (Li and Nicklas, 1995; Pinsky and Biggins, 2005; Thompson et al., 2010). However merotelic attachments are undetected by the SAC, potentially because they prevent an in-appropriate level of tension across the axis of the spindle (Gregan et al., 2011). In support of this, tumour cells that actively mis-segregate their chromosomes often have an active and functioning SAC (Thompson and Compton, 2008; Tighe, 2001). However merotelic attachments are frequently observed during early mitosis in normal cells but are rarely observed in late mitosis (Cimini, 2003). Therefore there must be a mechanism that actively repairs merotelic attachments.

Aurora B kinase has been shown to repair inappropriate MT kinetochore attachments and inhibition of this protein is sufficient to increase the frequency of merotelic attachments during mitosis (Cimini, 2006; Gregan et al., 2011). This is because active Aurora B destabilises inappropriately attached MTs at the kinetochores (Cimini, 2006). There is a gradient of active Aurora B that spreads across the kinetochore region. This is caused by the localisation of active Aurora B at the centromere and a high level of its corresponding phosphatase, protein phosphatase 1 (PP1) at the kinetochore (Kelly and Funabiki, 2009; Liu, 2010). This causes Aurora B activity to be highest in a region that is associated with inappropriately connected kinetochores. Increased tension on the kinetochore caused by inappropriate MT attachments changes the shape and/or orientation of the kinetochore – pulling it in to the region saturated with active Aurora B (Cimini, 2003; Cimini, 2006). The corner of the kinetochore attached to the wrong spindle pole is usually pulled in to this region and repaired – delivering the chromatid to the correct daughter cell (Figure 7) (Cimini, 2006; Welburn, 2010).
Figure 7. Repair of a merotelic attachment. Aurora B activity (in red) is saturated at the centromere. When a kinetochore is held in a merotelic attachment its shape is altered, pulling it in to the region saturated with active Aurora B. This promotes destabilisation of the inappropriately attached MT, resolving it to an amphitelic attachment. (Gregan et al., 2011).

However increasing the frequency of merotelic attachments during mitosis causes this machinery to fail (Cimini, 2001; Ganem, 2009; Silkworth, 2009). It has also been shown that defects in Aurora B signalling can stabilise MTs at the kinetochores, preventing repair of merotelic attachments (Bakhoum et al., 2009; Zhang et al., 2007). Unrepaired merotelic attachments result in lagging chromosomes at anaphase (Cimini, 2001). The segregation of a lagging chromosome is completely unregulated – it ultimately depends on which spindle pole releases the kinetochore first. Given this lagging chromosomes are a significant cause of genomic instability in tumour cells (Thompson and Compton, 2008).

1.10 The link between centrosome amplification, chromosomal instability and tumourigenesis

Centrosome clustering causes lagging chromosomes at anaphase by promoting merotelic attachments at the kinetochores, and lagging chromosomes are the most common defect found in tumour cells that actively mis-segregate their chromosomes (Ganem et al., 2009; Thompson and Compton, 2008; Thompson et al., 2010). Therefore lagging chromosomes are considered to be a major contributor to chromosomal instability (CIN). CIN is a dynamic process, it refers to the rate in which full chromosomes are mis-segregated following each round of mitosis.
Tumour cells that acquire a mechanism that actively promotes CIN can mis-segregate their chromosomes 10-100 times more frequently than tumour cells that lack any form of active CIN (Lengauer et al., 1997). Furthermore there is no bias towards which chromosomes are mis-segregated during CIN (Torosantucci et al., 2009). This means that a tumour cell with CIN can continually shuffle its chromosomes providing multiple different karyotypes per cell division. Therefore CIN can promote tumour heterogeneity (Cahill et al., 1999; Chandhok and Pellman, 2009). Acquiring a mechanism that promotes CIN is highly advantageous as this gives the tumour cell the ability to adapt to different environmental pressures. CIN has been shown to promote the clonal expansion of cells with features that give them a selective advantage within the surrounding environment (Gao et al., 2007; Nowell, 1976). Given this, CIN+ tumours have been associated with multi-drug resistance and poor patient prognosis (Carter et al., 2006; Choi et al., 2009; Heilig et al., 2009; Lee et al., 2011; Walther et al., 2008). Therefore understanding the mechanisms which actively promote CIN in tumour cells, such as centrosome clustering, is crucial for the development of new therapeutic agents for cancer treatment.

1.10.1 Mitotic defects and centrosome amplification

Defects during mitosis can promote the amplification of centrosomes. Events such as mitotic slippage and cytokinesis failure have been shown to promote supernumerary centrosomes. For example, over-expression of Aurora A promotes defective mitosis, specifically causing cytokinesis failure – resulting in centrosome amplification accompanied by polyploidy (Figure 8) (Anand et al., 2003; Meraldi et al., 2002).

However, tumour cells with supernumerary centrosomes have been shown to successfully complete mitosis (Basto et al., 2008; Ganem et al., 2009). Therefore centrosome amplification caused as a bi-product of defective mitosis will not be the only explanation for the observed frequency of supernumerary centrosomes in tumour cells. Furthermore cells that do not complete mitosis correctly do not segregate DNA between daughter cells. Therefore centrosome amplification caused due to an over-riding fault in mitosis is unlikely to actively promote CIN in tumour cells.
Figure 8. Centrosome amplification caused by cytokinesis failure. Following the successful completion of mitosis duplicated DNA and centrosomes are segregated evenly between two daughter cells. Cytokinesis failure prevents the segregation of components between daughter cells – producing a cell that is tetraploid and has supernumerary centrosomes. (Godinho and Pellman, 2014).
1.10.2 Uncoupling of the centriole cycle and the cell cycle

Centriole number is regulated by the centriole cycle. In normal cells the centriole cycle is tightly coupled to the cell cycle, ensuring centriole duplication is only licensed once during S-phase (Nigg and Stearns, 2011). The un-coupling of the centriole cycle from the cell cycle is sufficient to promote centrosome amplification (Figure 9). For example, inducing a G1/S-phase arrest in Chinese hamster ovary (CHO) cells is sufficient to cause centrosome amplification (Balczon et al., 1995). It was found that excessive re-duplication of centrosomes in response to a G1/S-phase arrest was dependent on the extended expression of CDK2 – a key regulator of centriole biogenesis (Matsumoto et al., 1999). Similarly over-expression of MPS1, a CDK2 target, is sufficient to promote centrosome amplification in this way. Therefore it was hypothesized that halting the cell cycle at this stage allowed the continual expression of key proteins required for centriole biogenesis – permitting excessive centriole duplication (Balczon et al., 1995; Matsumoto et al., 1999).

Figure 9. Centrosome amplification caused by uncoupling of the centriole cycle and the cell cycle. The centriole cycle follows the stepwise manner of the cell cycle, ensuring centriole duplication is initiated in S-Phase. In some cell lines cell cycle arrest has no effect on the progression of the centriole cycle. This allows centriole duplication to occur multiple times within the same cell – promoting centrosome amplification. (Brownlee and Rogers, 2013).
Similarly, inducing a G2/M phase arrest is sufficient to promote centrosome amplification. DT40 cells lacking Rad51 are incapable of repairing DNA lesions caused as a result of recombination – inducing a G2/M phase arrest (Dodson et al., 2004). This arrest was sufficient to promote centrosome amplification (Dodson et al., 2004). It was hypothesised that this may be the result of an internal DNA damage signal, inducing centrosome amplification which will promote multipolar spindle formation and selective cell death (Dodson et al., 2004). However an extended period in G2 may also permit the extended expression of key centrosomal proteins - permitting excessive centrosome duplication. It was later shown that extended G2 phase can induce premature centriole disengagement – licensing additional rounds of centriole duplication. This is due to the untimely expression of PLK1 and separase (Lončarek et al., 2010; Prosser et al., 2015).

Again this highlights the importance of the temporal regulation of proteins in maintaining normal centriole number – disruption of the regulation of proteins required for key steps in the centriole cycle is sufficient to promote centriole amplification.

Additional studies have identified factors that have a negative effect on centriole reduplication events – ie. they block additional rounds of duplication in arrested cells. Cep76 has been identified as a protein that has a negative effect on promiscuous centriole duplication events. Over-expression of Cep76 is sufficient to prevent centriole amplification caused as a result of G1/S-phase arrest (Matsumoto et al., 1999; Tsang et al., 2009). A similar phenotype was observed for MCM5, a protein primarily known for its role in limiting DNA replication to once per cell cycle (Ferguson and Maller, 2008). However the mechanism of action of these proteins remains unclear – they may down-regulate the expression of proteins required for centriole biogenesis or maintain the integrity of the S-M linker, preventing premature centriole disengagement.

It is clear that further study is required to fully understand the mechanisms by which centriole duplication is limited to once per cell cycle. However uncoupling of the centriole cycle and cell cycle permits the repeated duplication of centrioles. This results in centrosome amplification – a potential cause of CIN in tumour cells.
1.10.3 Unrestrained procentriole production

Copy number control is an additional mechanism ensuring a normal centriole number is maintained. Centriole duplication is semi-conservative, the mother centriole templates the formation of a single daughter procentriole (Kleylein-Sohn et al., 2007; Paintrand et al., 1992; Strnad et al., 2007). Loss of this copy number control has been shown to result in centriole amplification.

PLK4 is the master regulator of centriole biogenesis – this protein initiates a cascade of events that are essential for the assembly of procentrioles (Habedanck et al., 2005; Kleylein-Sohn et al., 2007). Over-expression of PLK4 is sufficient to induce centriole amplification and this was shown to promote tumourigenesis in flies (Basto et al., 2008). Notably, over-expression of PLK4 causes the formation of distinct rosette-like structures (Kleylein-Sohn et al., 2007). This represents a single mother centriole with several daughters (Figure 10).

Figure 10. Centrosome amplification caused by uncontrolled daughter centriole production. During S-phase the centrioles duplicate semi-conservatively (highlighted by red box) – a mother produces a single daughter procentriole (light green). When copy number control is lost a mother can assemble multiple daughters – resulting in centrosome amplification. (Brownlee and Rogers, 2013).
Over-expression of the components downstream of PLK4, for example SAS-6 or STIL, also induces the formation of these characteristic rosette-like structures (Arquint et al., 2011; Strnad et al., 2007). Notably the assembly of additional daughters relies not only on the over-expression of PLK4 but the abundance of additional downstream components. For example, SAS-6 is responsible for the assembly of the cartwheel base of newly formed procentrioles. Limiting the availability of SAS-6 will have consequences on the number of daughter centrioles assembled (Strnad et al., 2007).

Therefore copy number control also relies heavily on the precise regulation of expression of key proteins involved in centriole biogenesis. However this mechanism is molecularly distinct from centriole amplification caused as a result of uncoupling of the centriole and cell cycle – discussed in the previous section. For example, over-expression of Cep76 prevents centriole amplification caused as a result of G1/S-phase arrest, yet over-expression of Cep76 has no effect on centriole amplification driven as a result of ectopic PLK4 expression (Tsang et al., 2009). Taken together this reinforces that there are two molecularly distinct pathways involved in regulating centriole number – ensuring duplication occurs once per cell cycle and a single daughter centriole is produced per mother. Notably each of these processes relies heavily on the temporal expression of key centriolar components. Therefore the extensive de-regulation of several signalling pathways in tumour cells may be sufficient to promote expression changes which will alter the delicate balance of centriolar components, resulting in centriole amplification.

1.11 Therapeutic potential of the centriole cycle in cancer

As discussed, centrosome amplification is common in human tumours (Chan, 2011). Furthermore centrosome amplification promotes lagging chromosomes at anaphase, a major contributor to CIN (Thompson and Compton, 2008). Mechanisms which actively promote CIN are of great clinical significance as CIN is known to enhance tumour heterogeneity, this been linked to multi-drug resistance and poor patient prognosis (Carter et al., 2006; Choi et al., 2009; Heilig et al., 2009; Lee et al., 2011; Walther et al., 2008). Supernumerary centrosomes are only beneficial to tumour cells when they cluster in to a bipolar spindle, when clustering is inhibited this causes multipolar spindle formation – promoting intolerable levels of aneuploidy and ultimately cell death (Ganem et al., 2009).
Significantly, centrosome amplification is rarely observed in normal cells (Nigg, 2002; Pihan et al., 1998; Sato et al., 1999). This has identified a new therapeutic window for cancer treatment. As normal cells do not exhibit centrosome amplification there is no requirement of these cells to utilise the centrosome clustering pathway – a pathway that is fundamental for the survival of tumour cells with supernumerary centrosomes. HSET, a MT motor protein, has been shown to be crucial for centrosome clustering (Basto et al., 2008; Kwon et al., 2008). Notably HSET appears to be redundant within normal cells whilst remaining crucial for the survival of malignant cells (Ganem et al., 2009; Kwon et al., 2008). CW069, a HSET inhibitor, preferentially targets tumour cells with supernumerary centrosomes whilst having no effect on the viability of normal cells (Watts et al., 2013). This has identified a new potential inhibitor which could be utilised in a clinical setting. Therefore it remains essential to fully understand the mechanisms of regulation of the centriole cycle and how these processes become de-regulated in tumour cells to promote centrosome amplification. Deeper understanding of the centriole cycle may identify additional pathways that are exclusively utilised in tumour cells. Particularly as centrosome amplification is seen in a high proportion of tumour cells from varying tissues of origin – this may identify a common mechanism across multiple cancers that can be targeted by a single chemotherapeutic agent.

1.12 Tiam1 – a guanine nucleotide exchange factor

In this thesis I will be describing a novel role of the T-Cell lymphoma invasion and metastasis 1 protein (Tiam1) in the centriole cycle. Tiam1 is a large multi-domain protein that belongs to the Diffuse B cell lymphoma (Dbl)-like family of guanine nucleotide exchange factors (GEFs). It is ubiquitously expressed throughout adult tissues showing increased expression in the brain, testis and epidermis (Habets et al., 1994). As the name suggests, Tiam1 was first identified in T lymphoma cells (Habets et al., 1994). Using retroviral insertional mutagenesis, Tiam1 was identified as a protein that enhances the invasive potential of T lymphoma cell lines (Habets et al., 1994). Tiam1 can enhance the invasive potential of T lymphoma cells because it functions as a GEF.
The primary role of a GEF is to activate a small signalling molecule belonging to the Rho family of GTPases (Boissier and Huynh-Do, 2014). These signalling molecules cycle between an inactive-GDP form and an active-GTP bound form. When associated with GTP this causes a conformational change within the RhoGTPase, initiating a signalling cascade (Bourne et al., 1991). The RhoGTPases have been shown to regulate several diverse biological functions (Jaffe and Hall, 2005). Tiam1 specifically activates the small RhoGTPase called Rac (Michiels et al., 1995). Tiam1-mediated Rac activation causes re-arrangement of the cytoskeleton, therefore this signalling pathway mediates changes in cell morphology, adhesion and migration (Hall, 1998).

Tiam1 is a large multi-domain protein that is 1591 amino acids long (Figure 11). The N terminal region contains domains which are primarily involved in the regulation of the stability and localisation of Tiam1. The myristoylation site and two PEST (proline, glutamic acid, serine and threonine) domains that reside within the amino terminal are thought to regulate the stability of Tiam1. Removal of this region produces a mutant of Tiam1 (C1199) than is more stable and active than its full length counterpart (van Leeuwen et al., 1995). Downstream of this region is the PHn-CC-Ex domain which is important for regulating the interaction of Tiam1 with other proteins. It has been shown to facilitate interactions with the plasma membrane and other scaffolding proteins (Michiels et al., 1997; Stam et al., 1997; ten Klooster et al., 2006). However protein-protein interactions are not limited to this domain, several sites on Tiam1 have been shown to support protein interactions (Lambert et al., 2002; Mack et al., 2012; Shepherd et al., 2010). Downstream of the PHn-CC-Ex is the Ras binding domain and the PDZ (PSD-95/DlgA/ZO-1) domain (Boissier and Huynh-Do, 2014). Nestled within the C terminal region of Tiam1 is the DH-PH (PHc) domain. This domain is found within all members of the Dbl-family proteins (Habets et al., 1994). This region is responsible for the catalytic activity of Tiam1 and facilitates the exchange of GDP to GTP – crucial for mediating the activation of Rac (Tolias et al., 2005).
Tiam1 was first identified as a protein that promotes an invasive phenotype in T lymphoma cells due to the downstream activation of Rac (Habets et al., 1994). Further studies have replicated this invasive phenotype in additional cell lines – for example activation of Tiam1 promotes membrane ruffling and increased invasive potential in breast cancer cell lines (Adam et al., 2001). Therefore this would suggest that Tiam1-mediated Rac signalling behaves in an oncogenic manner. In support of this, knockout of Tiam1 has a protective effect in the initiation of tumourigenesis (Malliri et al., 2002). Tiam1 wild type and Tiam1 knockout mice were treated with a Ras-induced skin carcinogenesis protocol (Malliri et al., 2002). Mice lacking Tiam1 developed significantly less skin tumours when compared to wild type mice (Malliri et al., 2002). This correlated with an increase in apoptosis in the skin of Tiam1 knockout mice following treatment with carcinogens – this would have a negative effect on tumour initiation (Malliri et al., 2002). In support of this Tiam1-mediated Rac activation has been shown to protect keratinocytes from apoptosis (Rygiel et al., 2008). Therefore the Tiam1/Rac signalling pathway can promote cell survival. A similar protective phenotype was also observed in the intestines of mice (Malliri et al., 2006). When adding a driver to initiate tumourigenesis (mutation of APC) removal of Tiam1 reduced the tumour load observed within mice (Malliri et al., 2006). Activation of the Tiam1/Rac signalling pathway induces actin polymerisation at the cell membrane – promoting membrane ruffling and facilitating cell migration and invasion (Michiels et al., 1995). Over-expression of Tiam1 also enhances the migratory potential of colorectal cancer cells and this correlates with an enhanced tumour initiation phenotype within mice (Minard et al., 2005). Ectopic expression of Tiam1 has been implicated in human tumours and clinical prognosis.

1.12.1 Tiam1-mediated Rac activation and its role in tumourigenesis

Figure 11. Schematic of Tiam1. Tiam1 is a large scaffolding protein with multiple domains. PESTs: PEST sequence, PHnCCEx: N-terminal pleckstrin homology, coiled coil and extended domain, RBD: Ras binding domain, PDZ; PSD-95, Dlg1, ZO-1/2 domain, DH: Dbl homology domain, PHc: C-terminal pleckstrin homology domain, KETDI: KETDI amino acid sequence.
Tiam1 over-expression has been shown to correlate with poor patient prognosis in both esophageal squamous cell carcinoma and breast carcinoma (Adam et al., 2001; Liu et al., 2011; Minard et al., 2004). Therefore the activation of the Tiam1-Rac signalling cascade is heavily implicated in tumour initiation and progression.

However an interesting observation was made by Malliri and colleagues (2002). Although tumour burden was significantly reduced in Tiam1 knockout mice following treatment with a skin carcinogenesis protocol, a small number of tumours did develop and they were more likely to progress to malignancy when compared to tumours established in the wild type mice (Malliri et al., 2002). Therefore although knockout of Tiam1 reduces tumour initiation – loss of Tiam1 must aid tumour progression once a tumour has established. Tiam1-mediated Rac activation is important for the regulation of cell-cell adhesions in epithelial cells. Tiam1 and active Rac localise to adherens junctions where they positively reinforce E-cadherin mediated cell-cell adhesion complexes (Hordijk et al., 1997; Malliri et al., 2004). Loss of Tiam1 in an epithelial cell line promotes adherens junction disassembly and the cells present with a mesenchymal morphology – potentiating cell migration (Malliri et al., 2004). The enhanced malignant phenotype observed in the few tumours established in the Tiam1 knockout mouse could be due to the role of Tiam1 in mediating cell-cell adhesion (Malliri et al., 2002). Therefore ectopic Tiam1 expression within an established tumour could provide a suppressive force to malignant progression.

Therefore the role of Tiam1-mediated Rac activation in tumourigenesis remains complex. It is clear that Tiam1 is required for optimal tumour formation, however the role of Tiam1 in tumour progression remains a challenging question. Loss of Tiam1 in the early stages of tumourigenesis may be beneficial as this increases apoptosis (Malliri et al., 2002; Rygiel et al., 2008). However epithelial tumours that develop in the absence of Tiam1 - due to the de-regulation of another oncogenic pathway – could benefit from having no Tiam1 as this could promote adherens junction disassembly and cell migration (Hordijk et al., 1997; Malliri et al., 2002; Malliri et al., 2004). Therefore interpreting the effect of Tiam1 expression in tumour initiation and progression remains complex and context dependent.
1.12.2 Tiam1 and the centriole cycle

Tiam1-mediated Rac activation has been shown to regulate centrosome separation during mitosis (Woodcock et al., 2010). Tiam1 localises to the centrosomes in MDCK cells where it counteracts the action of Eg5, a MT motor protein – the collective force generated by Eg5 and Tiam1 promotes bipolar spindle assembly (Woodcock et al., 2010). Further studies have shown that phosphorylation of Tiam1 by CDK1 – a potent initiator of mitotic progression – is crucial for the activation of Tiam1 at the centrosomes and its ability to regulate centrosome separation (Whalley et al., 2015).

Activation of Tiam1 by CDK1 promotes the downstream activation of Pak1 and Pak2, proteins that collectively function to antagonise the action of Eg5 – promoting centrosome separation (Whalley et al., 2015).

During these studies it was observed that down-regulation of Tiam1 in a colon tumour cell line correlated with an increase in centriole number, a process that is distinct from centrosome separation. Based on this preliminary data and the fact that no studies to date have linked Tiam1 or Rac to regulation of centriole number, the role of Tiam1-mediated Rac signalling in the centriole cycle was investigated.

1.13 Project Hypothesis

Tiam1 is a novel regulator of the centriole cycle.

1.13.1 Project rationale

Tiam1 is a GEF that specifically activates the small Rho GTPase Rac (Michiels et al., 1995). The Tiam1/Rac signalling cascade has been heavily implicated in tumourigenesis due to its roles in cellular migration, adhesion and cell survival (Mack et al., 2011). The Tiam1/Rac signalling pathway has also been implicated in the regulation of centrosome separation by antagonising the action of Eg5 (Whalley et al., 2015; Woodcock et al., 2010). The aim of this project was to determine whether Tiam1 has a novel role in regulating centriole number.
Thorough characterisation of novel proteins involved in the regulation of the centriole cycle could uncover novel markers that could be used to identify tumours with centrosome amplification. As tumour cells tolerate centrosome amplification by centrosome clustering, preventing centrosome clustering can cause selective death of tumour cells by promoting multipolar spindle formation and intolerable levels of aneuploidy (Watts et al., 2013). Therefore determining the functional role of Tiam1 in the centriole cycle could provide evidence that Tiam1 is a suitable marker of centrosome amplification and could identify tumours that would benefit from treatment with centrosome de-clustering drugs.
Chapter 2. Materials and Methods

2.1 Buffers and Solutions

Cell Freezing Medium  50% FBS (v/v), 40% culture medium lacking antibiotics (v/v), 10% DMSO (v/v)
Cell Permeabilisation Buffer  0.1% Triton (v/v) in PBS
Dialysis Buffer  50mM Tris, pH 7.5, 5mM MgCl, 5mM βMercaptoethanol
FACS Blocking Buffer  0.5% BSA (w/v), 0.1% Triton (v/v) in PBS
FACS Wash Buffer  0.5% BSA (w/v) in PBS
GST Elution Buffer  1.54g Glutathione and 0.2g NAOH dissolved in 50 ml ddH2O, then add 50mM Tris pH 8.0, 0.5M NaCl and protease inhibiting tablets (Roche)
GST Lysis Buffer  0.5M NaCl, 1mM EDTA, 2mM Benzamidine, adding fresh protease inhibitor tablet (Roche)
GST Wash Buffer  0.5M NaCl, 1mM EDTA, 0.25% Triton-X (v/v)
IF Blocking Buffer  1% BSA (w/v) in PBS
IP Blocking Buffer  50% BSA (w/v) in PBS
IP Wash Buffer  50mM Tris-HCL, pH 7.5, 150mM NaCl, 1% Triton-X-100 (v/v), 10% glycerol (v/v), 2mM EDTA, 25mM NaF and 2mM NaH2PO4 in ddH2O
PBS  137mM NaCl, 2.7mM KCl, 10mM Na2PO4, 2mM KH2PO4
PBS/Tween  0.1% Tween (v/v) in PBS
PI Solution  50µl/ml PI (Sigma), 0.1% Triton (v/v), 10ug/ml RNAse in PBS
Protein Lysis Buffer  50mM Tris-HCL, pH 7.5, 150mM NaCl, 1% Triton-X-100 (v/v), 10% glycerol (v/v), 2mM EDTA, 25mM NaF and 2mM NaH2PO4, adding fresh protease inhibitor tablet (EDTA free, Roche) and phosphatase inhibitor cocktails 2 and 3 (Sigma) in ddH2O
SDS PAGE Sample Buffer | 2x NuPAGE® LDS sample buffer (Invitrogen), NuPAGE® sample reducing agent (Invitrogen) 
---|---
TAE Buffer | 40mM Tris, 20mM acetic acid, 1mM EDTA 
Western Blocking Buffer | 5% Milk Powder (w/v) in PBS/Tween 
Western Stripping Buffer | 0.2M glycine, 1% SDS (v/v), HCL, pH2.5 

2.2 Cell Culture Techniques 

2.2.1 Cell Maintenance 
All cell lines were cultured at 37°C in a humidified incubator (5% CO₂ atmosphere). U2OS, HCT116, Phoenix GP and HEK 293 cells were cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco). MCF7 cells were cultured in DMEM (Sigma) supplemented with 10% FBS, 1% L-Glutamine (Gibco). 

To passage, cells were washed once in PBS and incubated with trypsin (Gibco) at 37°C until suitably detached. Trypsin was inactivated by the addition of serum and cells were plated at an appropriate density. For experiments cell concentrations were determined using a Coulter Counter (Beckman Coulter).

2.2.2 Long Term Storage of Cell Lines 
Cells were grown to confluency in a T225 flask (Corning). Cells were detached by trypsinsation as described (Section 2.2.1), pelleted by centrifugation and resuspended in Cell Freezing Medium. Cells were then stored in liquid nitrogen.

2.2.3 Cell Transfection 
Cells were plated at a density of 1x10⁵ cells/ml of culture media. siRNA oligos were transfected following the siRNA Transfection Protocol (DharmaFECT Transfection Reagents, Dharmacon) whilst plasmids were transfected using Transit-LT1 Transfection Reagent (Mirus) following the manufacturers protocol.

2.2.4 Retrovirus Production 
Pheonix GP cells were plated at a density of 1x10⁵ cells/ml in a 10cm dish. 3µg of desired plasmid and 3µg of packaging VSVG plasmid were transfected with Transit-LT1 Transfection Reagent as described (Section 2.2.3).
Fresh DMEM was added to Phoenix GP cells 12 hours post transfection and virus was collected at 20 and 32 hours, respectively. Media containing virus was centrifuged at 1200 rpm for 5 minutes and passed through a 0.45µm filter. Polybrene (8µg/ml) was added to filtered virus and added to target cells plated at a density of 1x10⁴ cells/ml.

2.2.5 Production of Inducible Cell Lines
The pRetroX-Tight system (Clontech) inducibly expresses proteins upon the addition of DOX. U2OS cells were retrovirally transduced (Section 2.2.4) with pRetroX-Tet-On and grown in G418 media for 7 days to select for cells containing the plasmid. U2OS pRetroX-Tet-On cells were retrovirally transduced (Section 2.2.4) with the second plasmid, pRetroX-Tight-Pur, and continually grown in G418/puromycin media to maintain expression of both plasmid vectors. Using this method U2OS cells were made which exogenously express Tiam1 WT, Tiam1 GEF* and Tiam1 Δ25 following the addition of DOX (Table 4; Section 2.3.10b)

2.3 Molecular Biology Techniques
2.3.1 PCR
50ng of template and 0.5µl of primer (10mM) were added to a HF Polymerase reaction mix (ThermoFisher) as described by the manufacturer. Template was amplified by the following protocol:
Step 1: Denature (initial), 98°C, 30 seconds
Step 2: Denature, 98°C, 10 seconds
Step 3: Anneal, 69°C, 30 seconds
Step 4: Extension, 72°C, 3 minutes
Repeat steps 2-4 29 times
Step5: Extension (final), 72°C, 10 minutes

2.3.2 Restriction Digests
0.5µg of DNA was digested using 0.5µl of required restriction enzyme with recommended restriction enzyme buffer, diluted in 20µl of ddH2O. The digest mix was incubated at 37°C for 2 hours. If re-annealing was a possibility the digest mix was incubated for a further 30 minutes with alkaline phosphatase (1µl alkaline phosphatase: 2µl ddH2O).
2.3.3 Agarose Gel Electrophoresis
DNA was added to DNA loading buffer (Promega) and ran on 1% agarose/TAE gel containing Gel Red (1:10000, v/v) (Sigma) to allow visualisation of DNA by UV (205nm).

2.3.4 DNA Extraction
DNA was cut from agarose gel and extracted using the QIAquick gel extraction kit (Qiagen) following the manufacturers protocol.

2.3.5 Ligation of Restriction Digest in to Plasmid Backbone
T4 DNA ligase (Life Technologies) was added to varying ratios of vector: insert (1:1; 1:2; 1:4) in a reaction mix recommended by the manufacturer. Ligation reactions were incubated at RT for 3 hours.

2.3.6 Quantification of DNA Concentration
DNA concentration was determined using the NanoDrop instrument (ThermoFisher).

2.3.7 Transformation of Competent Bacteria
2µl of target plasmid was transformed in to 23µl of One ShotR TOP10 competent cells (Invitrogen) following the manufacturers protocol. This protocol was also used to transform target plasmids in to Rosetta competent cells kindly donated by Professor Karim Labib (University of Dundee). Bacteria were spread on to agar plates containing the appropriate antibiotic selection and grown at 37°C overnight. Single colonies were selected for further analysis.

2.3.8 Obtaining DNA from Competent Bacteria
Single bacterial colonies were added to 5ml of L-Broth (containing suitable antibiotic) and grown at 37°C overnight with agitation. The mini prep procedure was carried out by the Molecular Biology Core Facility at the CRUK Manchester Institute. To obtain higher concentrations of target DNA the 5ml overnight culture was added to 400ml of L-Broth (containing suitable antibiotic) and grown at 37°C overnight with agitation. DNA was obtained using the QIAquick Plasmid Maxi Kit (Qiagen) following the manufacturers protocol.
2.3.9 DNA Sequencing
To obtain sequencing data, 0.5µg of DNA was added to 15pmol of sequencing primer and
diluted to a final volume of 12µl in nuclease free H2O (Life Technologies). Sequencing
reactions were carried out by the Molecular Biology Core Facility at the CRUK
Manchester Institute.

Table 1. Details of sequencing primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEXFOR</td>
<td>GCATGGCCTTTTGCAGGGCTG</td>
</tr>
<tr>
<td>pGEXREV</td>
<td>TGACCGTCTCCGGAGCTGC</td>
</tr>
<tr>
<td>pRetroREV</td>
<td>GAAGGGGCCACCAAAGAACGG</td>
</tr>
<tr>
<td>pTight</td>
<td>ATCTGAGGGCCCTTTCTTCCTTCACT</td>
</tr>
</tbody>
</table>

2.3.10 Generation of Expression Vectors

2.3.10a MCM5-GST
MCM5 cDNA was PCR amplified (Section 2.3.1) from pCMV-Sport6 (clone ID: 2900229; ThermoFisher; Table 4) using the following primers;
Forward:
5’ – CCCCGGATCCTCGGGATGACGAT – 3’
Reverse:
5’ – CCCCGGCGCCCGCGCTACTTGAGGCAGGAGTAGAAG – 3’
These primers introduce a BamHI site 5’ and a stop codon followed by a NotI site 3’. The MCM5 PCR fragment was ligated in to pGEX4T1 by digesting the vector
and insert with BamHI and NotI (Section 2.3.5). Positive clones were identified by
sequencing with pTight and pRetroREV sequencing primers (Section 2.3.9).

2.3.10b Tiam1 Δ25
The 25 amino acid region identified by Professor George Bailie (Glasgow
University) was removed from pRetroXT Tiam1 WT-HA by PCR (Section 2.3.1; Table 4). Primers were designed following guidelines provided by the
Quickchange XL Site Directed Mutagenesis Kit (Agilent);
Forward:
5’ – CTCCCTGGGTGACTTGGTGTCGGATATTCCCG – 3’
Reverse:
5’ – CGGGAATATCCGACACCAAGTCACCCAGGAG – 3’
Positive clones were identified by sequencing with pGEXFOR and pGEXREV sequencing primers (Section 2.3.9). The mutagenesis protocol and selection of positive clones was carried out by Gavin White.

2.3.11 Expression of MCM5-GST in competent bacteria
MCM5-GST was transformed into Rosetta competent bacteria (Section 2.3.7) and grown in a shaker overnight at 37°C. The bacterial culture was diluted 1:40 and grown at 30°C until the OD at 600nm was between 0.6-0.8 (measurement taken on a UV/Visible spectrophotometer). Expression of MCM5-GST was induced by adding 1mM IPTG (Sigma) and culturing overnight at 24°C with agitation.

2.3.11a Purification of MCM5-GST from competent bacteria
Rosetta bacteria expressing MCM5-GST were centrifuged at 6 k rpm for 10 minutes, 4°C. The pellet was resuspended in 10mls of GST Lysis Buffer and sonicated at 10Amp. 1% Triton-X (v/v) was added to lysate and rotated at 4°C for 30 minutes. The lysate was centrifuged for 2 hours at 13 k rpm, 4°C, and mixed with Glutathione beads (GE Healthcare) for 2 hours at 4°C. MCM5-GST bound beads were washed 5x with GST Wash Buffer and added to a chromatography column (Biorad). MCM5-GST was eluted from beads by incubating for 30 minutes at RT with GST Elution Buffer. Elutant was added to a Slide-A-Lyzer™ dialysis cassette (20kDa) (ThermoFisher) and dialysed overnight in Dialysis Buffer. MCM5-GST protein was concentrated using Ultra Amicon Centrifugal Filter Units (50kDa) (Millipore).

2.4 Protein Detection Techniques
2.4.1 Protein Lysates
Protein was obtained from cultured cells by washing twice in ice cold PBS and scraping cells into an appropriate volume of Protein Lysis Buffer. Collected cells were incubated on ice for 5 minutes and protein lysate was isolated by centrifugation at 4°C for 10 minutes at 13 k rpm.
2.4.2 Quantification of Protein Concentration
Protein concentrations were determined by diluting protein lysate in Advanced Protein Assay Reagent (Cytoskeleton Inc.) following the manufacturers guidelines. Absorption readings were taken at 595nm using a UV/Visible spectrophotometer.

2.4.3 SDS-PAGE and Immunoblotting
Protein lysates prepared to appropriate loading concentrations were diluted in SDS-PAGE Sample Buffer (Invitrogen) following the manufacturers guidelines and heated to 70°C for 10 minutes. Samples were run on 3-8% NuPAGE gels (Invitrogen) using the XCell SureLock™ Mini-Cell electrophoresis system. To confirm protein molecular weight all samples were run alongside the Rainbow Full Range Molecular Weight Marker (GE Healthcare). Proteins were transferred to PVDF membranes (Immobilon-P, Millpore) which were activated by soaking in 100% methanol using the XCell SureLock™ transfer system. Membranes were incubated overnight at 4°C in Western Blocking Buffer. Membranes were rinsed with PBS/Tween and incubated with primary antibody diluted in Western Blocking Buffer for 1 hour at RT on a rocker. Membranes were briefly rinsed 6x with PBS/Tween, followed by 3x 10 minute washes with PBS/Tween at RT on a rocker. Membranes were then incubated for 1 hour at RT with the appropriate HRP-conjugated secondary antibody (GE Healthcare) diluted in Western Blocking Buffer. Washes in PBS/Tween were repeated as described. Immunoblots were then visualised using ECL (Perkin-Elmer).

To re-probe, blots were incubated with Western Stripping Buffer for 20minutes and blocked for 1 hour in Western Blocking Buffer. Antibodies were then applied as described above.
Table 2. Antibodies used for immunoblotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Manufacturer</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Rabbit-HRP</td>
<td>Rabbit</td>
<td>GE Healthcare</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-Mouse-HRP</td>
<td>Mouse</td>
<td>GE Healthcare</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-Sheep-HRP</td>
<td>Sheep</td>
<td>GE Healthcare</td>
<td>1:5000</td>
</tr>
<tr>
<td>β Actin</td>
<td>Mouse</td>
<td>Cancer Research UK</td>
<td>1:5000</td>
</tr>
<tr>
<td>GST-HRP</td>
<td>Goat</td>
<td>Abcam</td>
<td></td>
</tr>
<tr>
<td>HA (12CA5)</td>
<td>Mouse</td>
<td>Sigma</td>
<td>1:5000</td>
</tr>
<tr>
<td>HA (12CA5)</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>1:5000</td>
</tr>
<tr>
<td>MCM5 (CRCT5.1)</td>
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<td>AbD Serotec</td>
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<tr>
<td>Tiam1</td>
<td>Rabbit</td>
<td>Bethyl</td>
<td>1:1000</td>
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<tr>
<td>Tiam1</td>
<td>Sheep</td>
<td>R&amp;D Systems</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

2.4.4 Immunoprecipitation (IP)

To detect protein-protein interactions, GammaBindG-Sepharose beads (GE Healthcare) were blocked overnight at 4°C in IP Blocking Buffer and washed 4x with IP Wash Buffer. Appropriate antibody was bound to 20μl of pre-blocked beads by incubating at RT for 1 hour. Antibody bound beads were washed 4x with IP Wash Buffer to remove residual antibody. Pre-prepared lysate was incubated with antibody-bound beads for 2 hours at 4°C. Unbound lysate was removed by washing 4x with IP Wash Buffer. Proteins bound to beads were eluted by the addition of SDS-PAGE sample buffer (Invitrogen) and incubating at 70°C for 10 minutes. Samples were analysed by immunoblotting as described (Section 2.4.3).
Table 3. Antibodies used for IP

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Manufacturer</th>
<th>Concentration (µg/IP)</th>
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<td>Tiam1</td>
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<tr>
<td>HA (12CA5)</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>0.5</td>
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</tbody>
</table>

2.5 Cell Imaging Techniques

2.5.1 Cell Fixation

Cells seeded to coverslips were fixed by:

- Formaldehyde Fixation: cells were incubated with 3.7% formaldehyde/PBS for 30 minutes at RT.
- Methanol Fixation: cells were incubated with pre-chilled 100% methanol for 5 minutes at -20°C.

Following fixation cells were washed 3x in PBS and stored at 4°C.

2.5.2 Immunofluorescence (IF)

Cells fixed to coverslips (Section 2.5.1) were incubated for 3 minutes with Cell Permeabilisation Buffer and blocked for 1 hour in IF Blocking buffer. Primary antibody diluted in IF Blocking Buffer was added for 1 hour at RT and coverslips were washed with 3x PBS to remove unbound antibody. Following this, secondary antibody diluted in IF Blocking Buffer was added for 1 hour at RT (in the dark) and subsequently washed 3x in PBS. Coverslips were mounted to microscope slides using Antifade solution containing DAPI (Molecular Probes). Slides were stored at RT in the dark.

2.5.2a IF using same-species antibodies

When same species antibodies were required for an experiment, the IF protocol was followed as described above (Section 2.5.2). Following the addition of secondary antibody, coverslips were incubated with same-species normal IgG diluted in IF Blocking Buffer for 1 hour at RT.
Coverslips were washed 3x in PBS and incubated with same-species Fab Fragments (Jackson), diluted in IF blocking buffer, for 1 hour at RT. Following 3x washes in PBS the IF protocol (Section 2.5.2) was repeated for the additional same-species primary antibody.

Table 4. Antibodies used for IF

<table>
<thead>
<tr>
<th>Antibody</th>
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<td>Jackson</td>
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<td>Rabbit</td>
<td>Jackson</td>
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<tr>
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<td>Invitrogen Molecular Probes</td>
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</tr>
<tr>
<td>Tiam1</td>
<td>Sheep</td>
<td>R&amp;D Systems</td>
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2.6 Microscopy

2.6.1 Spinning Disc
The Spinning Disc confocal is based on an Olympus IX81 microscope and uses an Andor iXon DU888+ camera. Fluorescence is achieved by an array of imaging lasers (406, 488, 548, 645nm). The system utilises Metamorph to capture and process images. Images were taken using the 100x oil lense.

2.6.2 Deltavision Core
The Deltavision Core system is based on an Olympus IX71 microscope. The system uses a Roper Cascade 512B camera and fluorescence is achieved by a 300W Xenon light source which has an array of Sedat filter sets (406/488/568/647nm). The system utilises SoftWorx to capture and process images. Images were taken using the 100x/60x oil lense.

2.6.3 Low Light
The Low Light system is based on a Zeiss Axiovert 200M microscope. The system uses an Andor iXon DU88+ camera and fluorescence is achieved by a 300W Xenon light source with an array of ET-Sedat filters (406/488/568/647nm). The system utilises Metamorph to capture and process images. Images were taken using the 100x oil lense.

2.6.4 The Leica GatedSTED SP8 (gSTED)
This super resolution microscope has been developed using stimulated emission depletion technology. The lateral resolution of an image is improved to 30nm (the lateral resolution of standard confocal microscopy ~200nm) by using two imaging lasers, an excitation laser and a depletion laser. When used in tandem this limits the volume of light which is emitted from a fluorescent marker, in turn increasing the resolution. This system utilises LAS AF Lite software (Leica) to capture and process images. Images were taken using the 60x oil lense.

2.7 Determining the Effect of Tiam1 Knockdown on Centriole Number

2.7.1 Tiam1 knockdown by siRNA
U2OS, HCT116 and MCF7 tumour cell lines were collected by trypsinisation at a density of 1x10^5 cells/ml. Tiam1 was knocked down by siRNA (Section 2.2.3) using the following oligos; Tiam1 #1 siRNA (5’ – GAGGTTGCAGATCTGAGCA – 3’) and Tiam1 #2 siRNA (5’ – GGTTCTGTCTGCCCCAATAA – 3’) whilst cells remained in suspension.
The cell suspension/transfection mix was seeded to coverslips and cultured for 72 hours, with fresh DMEM added 24 hours post transfection.

2.7.2 Detection of centrioles

Cells were fixed in methanol (Section 2.5.1) and centrioles were visualised by IF (Section 2.5.2) using:

- Centrin as a marker of centrioles;
  Centrin 20H5 (Millipore) was used to highlight the centriole structure while the pericentriolar material was detected by pericentrin (Abcam).
- Cp110 as a marker of centrioles;
  Cp110 (Proteintech) was used to highlight the centriole structure in addition to a second marker of centrioles, Centrin 20H5 (Millipore).

2.7.3 Quantification of centriole number

Centriole number was quantified from 0.1µm stacked images taken on the Spinning Disc and Deltavision Core microscopy systems – images were viewed using Image J software. When using the gSTED microscopy system single plane images were taken and analysed using LAS AF Lite software (Leica Microsystems). Single cells containing ≥5 centriole signals were classed as aberrant. Statistical significance was calculated using Prism software (unpaired t test; two-tailed).

2.8 Assays of Centriole Functionality

2.8.1 Depolymerisation of the cytoplasmic microtubule network

Tiam1 was transiently depleted in U2OS cells by siRNA and seeded to coverslips as described (Section 2.7.1). 72 hours post transfection cells were transferred to pre-cooled DMEM (4°C) and incubated on ice for 30 minutes to depolymerise cytoplasmic microtubules.

- For detection of centriolar satellites;
  Cells were fixed in methanol (Section 2.5.1) immediately following 30 minute incubation on ice and centrioles were visualised by IF using centrin as described (Section 2.7.2) α-Tubulin-FITC (Sigma) was also used to visualise microtubules. Centriole number was quantified using the Deltavision Core microscope as described (Section 2.7.3).
• For microtubule regrowth assay;
  4°C DMEM was replaced with pre-heated 37°C DMEM and cells were incubated at
  37°C for 20 seconds. Centrioles were visualised by IF using centrin as described
  (Section 2.7.2). α-Tubulin-FITC (Sigma) was also used to detect microtubules.
  Microtubule regrowth was assessed using the gSTED microscopy system. Single
  plane images were taken and analysed using LAS AF Lite software (Leica
  Microsystems).

2.9 Determining the Mechanism of Centriole Amplification

2.9.1 Cell cycle analysis
U2OS tumour cells were plated at a density of 1x10^5 cells/ml and Tiam1 was knocked
down by siRNA for 72 hours as described (Section 2.7.1). Cells were trypsinised and
pelleted by centrifugation (1200 rpm, 5 minutes). Pelleted cells were re-suspended in PBS.
Cells were fixed by adding 100% ethanol (-20°C) dropwise whilst gently vortexing. Fixed
cells were stored at -20°C for a minimum of 6 hours before staining with propidium iodide
(PI). To stain with PI fixed cells were centrifuged (1200 rpm, 5 minutes), washed once in
PBS and re-suspended in PI Solution. Stained cells were incubated in PI Solution for 30
minutes at RT before an additional incubation at 4°C for a minimum of 6 hours prior to
analysis. Cell cycle profiles were determined using the LSRII flow cytometer (BD
Biosciences) and data was analysed using FloJo software.

2.9.2 Tiam1 over-expression and hydroxyurea arrest

2.9.2a Hydroxyurea treatment
U2OS tumour cells exogenously expressing various Tiam1 constructs were seeded to
coverslips at a density of 6x10^4 cells/ml 24 hours prior to treatment. Cells were treated with
4mM hydroxyurea (Sigma) for 96 hours.

2.9.2b Exogenous expression of Tiam1
• Constitutive;
  U2OS cells were stably transfected with pEGFP-Tiam1 WT and pEGFP-Empty
  Vector using LT1 transfection reagent (Mirus) as described (Section 2.2.3; Table
  4). Exogenous expression was maintained by culturing cells in appropriate selective
  antibiotic (G418).
Following hydroxyurea treatment, these cells were fixed in 3.7% formaldehyde (Section 2.5.1) and stained for centrioles using centrin (Section 2.7.2). In addition to this GFP antibody (Abcam) was used (overnight incubation at 4°C) following the FAB fragment protocol (Section 2.5.2a) to detect GFP positive cells for the analysis. Centriole number was quantified from images taken on the Spinning Disc microscope as described (Section 2.7.3).

- Inducible;
  U2OS which inducibly over-express HA tagged Tiam1 WT, Tiam1 GEF* and Tiam1 Δ25 were produced by retroviral transduction (Section 2.2.5; Section 2.3.10b; Table 4). Exogenous expression was induced by the addition of DOX (1:1000) 8 hours prior to treatment with hydroxyurea. Fresh DOX was added every 48 hours. Post hydroxyurea treatment cells were fixed in methanol (Section 2.5.1) and stained for centrioles using centrin (Section 2.7.2). HA antibody (Abcam) was used (overnight incubation at 4°C) following the FAB fragment protocol (Section 2.5.2a) to allow selection of high expressing cells for the analysis. Centriole number was quantified from images taken on the Deltavision Core microscope as described (Section 2.7.3).

2.10 Confirming the Tiam1-MCM5 Interaction
2.10.1 IP of endogenous Tiam1

U2OS cells were seeded at a density of 6x10^4 cells/ml and treated with thymidine (18 hours 2mM thymidine; 8 hours release; 17 hours 2mM thymidine) (Sigma). Control (no treatment) and thymidine treated cells were collected by mechanical scraping and pelleted by centrifugation (1200 rpm, 5 minutes). Subsets of cells were prepared for cell cycle analysis as described (Section 2.9.1) to confirm partial cell cycle arrest by thymidine. HCT116 cells were seeded at a density of 6x10^4 cells/ml and collected by mechanical scraping when cells appeared 80% confluent.

Protein lysates of U2OS and HCT116 were prepared (Section 2.4.1) and pre-cleared overnight by incubating with 15µl of GammaBindG-Sepharose beads (GE Healthcare) at 4°C. Tiam1 was precipitated from lysate following the IP protocol outlined in Section 2.4.4. 1µg of Tiam1 antibody (R&D Systems) was used per sample. Beads bound with 1µg of IgG were used as a negative control.
2.10.2 GST \textit{in vitro} assay
Glutathione beads (GE Healthcare) were washed 4x with GST Wash Buffer. MCM5-GST purified from bacteria (Section 2.3.11) was bound to washed glutathione beads (10µl per reaction) by incubating at RT for 1 hour. Equal volumes of HECT-GST and GST only protein were used as controls for the \textit{in vitro} reaction. Antibody bound beads were washed 4x with GST Wash Buffer and incubated with equal volumes of purified Tiam1-HIS protein for 2 hours at 4°C. Unbound protein was removed by 4x washes with GST Wash Buffer. Proteins bound to beads were eluted by the addition of SDS-PAGE sample buffer. Samples were analysed by immunoblotting as described (Section 2.4.3).

2.11 Mapping the MCM5 binding region
2.11.1 IP of Tiam1 truncation mutants
HEK 293 cells were seeded at a density of 1x10^5 cells/ml and transfected with the following pcDNA3.1 constructs using LT1 transfection reagent (Section 2.2.3); Tiam1 WT-HA, C1199-HA, C580-HA, C196-HA, KETDI44-HA, M4-HA, PHCCEx-HA (Table 4). Empty pcDNA3.1 was used as a negative control. Cells were collected by mechanical scraping 48 hours post transfection and protein lysates were prepared as described (Section 2.4.1). Protein lysates were pre-cleared overnight by incubating with 15µl of GammaBindG-Sepharose beads (GE Healthcare) at 4°C. Tiam1 constructs were precipitated from lysate using the HA tag. 0.5µg of HA antibody (Abcam) was used per sample following the IP protocol outlined in Section 2.4.4.

2.11.2 IP of Tiam1 Δ25
U2OS cells that inducibly express Tiam1 Δ25 and Tiam1 WT were generated using the pRetroX-Tight-pur system outlined in Section 2.2.5. These cells were seeded to a density of 1x10^5 cells/ml and expression of each construct was induced by the addition of DOX (1:1000). Asynchronous and thymidine blocked cells (18 hours 2mM thymidine; 8 hours release; 17 hours 2mM thymidine) were collected by mechanical scraping following 3 days treatment with DOX and protein lysates were prepared as described (Section 2.4.1). Protein lysates were pre-cleared overnight by incubating with 15µl of GammaBindG-Sepharose beads (GE Healthcare) at 4°C. Endogenous MCM5 was precipitated from pre-cleared lysate following the IP protocol outlined in Section 2.4.4. 0.5µg of MCM5 antibody (AbD Serotec) was used per sample. Beads bound with 0.5µg of IgG were used as a negative control.
2.11.3 MCM5 knockdown by siRNA
U2OS were collected by trypsinisation at a density of 1x10^5 cells/ml. Tiam1 was knocked down by siRNA (Section 2.2.3) using the following oligos; MCM5 #1 siRNA (5’ – GGATGAACTCAAGCGGCA – 3’), MCM5 #2 siRNA (5’ – GCCTGAAGTCGGACATGA – 3’) and MCM5 #3 siRNA (5’ – GTAGCCTGAAGTCGGACA – 3’) whilst cells remained in suspension. The cell suspension/transfection mix was cultured for 72 hours, with fresh DMEM being added 24 hours post transfection. Protein lysates were prepared (Section 2.4.1) and protein levels were analysed by immunoblotting as described (Section 2.4.3).

2.12 Identification of Proteins Localised at the Centrosome
2.12.1 Detection of endogenous Tiam1 and MCM5 at the centrioles
U2OS cells were seeded to coverslips at a density of 1x10^5 cells/ml 24 hours prior to fixation in methanol (Section 2.5.1). Localisation of Tiam1 and MCM5 was determined by IF using centrin (Millipore) as a marker of the centrioles (Section 2.5.2). To detect endogenous Tiam1 and MCM5, both antibodies were incubated over night at 4°C (Tiam1; R&D Systems, MCM5; Santa Cruz). The localisation of each protein was examined on the Low Light microscopy system.

2.12.2 Localisation of Tiam1 and MCM5 in Tiam1 knockdown tumour cells
2.12.2a siRNA knockdown
Tiam1 was transfiently depleted by siRNA as described (Section 2.7.1). Tiam1 and MCM5 were visualised at the centrioles by IF as described in Section 2.12.1.

2.12.2b CRISPR clone
U2OS tumour cells which are heterozygous for Tiam1 (U2OS Tiam1 +/-) were generated by Gavin White in the laboratory using CRISPR technology. To avoid any compensatory effects, U2OS were retrovirally transduced with pRetroXT Tiam1 WT-HA (Section 2.2.5; Table 4) to inducibly express Tiam1 upon the addition of DOX. U2OS Tiam1 +/- were continually cultured in the presence of DOX (1:20000).
2.12.3 Detection and quantification of Tiam1 and MCM5 signal at the centrioles

U2OS Tiam1\textsuperscript{+/-} cells generated in Section 2.12.2b were cultured in the absence of DOX for a minimum of 3 passages to prevent any exogenous expression of Tiam1. Cells were seeded to coverslips 24 hours prior to fixation in methanol (Section 2.5.1) at a density of 1x10\textsuperscript{5} cells/ml. Tiam1 and MCM5 proteins were detected by IF as described (Section 2.12.1). 0.2µm stacked images were taken using the Deltavision Core microscopy system. The fluorescent intensity of Tiam1 and MCM5 was measured from these images using Image J software. A region of interest was selected around the centrioles and the total levels of light intensity within this region were measured. Intensity values obtained from Tiam1 over-expressing cells were normalised to the average fluorescent intensity measured from control cells (U2OS Tiam1\textsuperscript{+/-}).

Table 5. Details of supplied Constructs

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Chapter 3. Tiam1 Regulates Centriole Number

3.1 Tiam1 knockdown increases centriole number

3.1.1 Introduction

The centriole cycle is tightly regulated in normal, untransformed cells as aberrant centriole number has been linked to spindle multipolarity and decreased cell viability (Ganem et al., 2009). The centriole cycle is tightly coupled to the cell cycle, ensuring centriole biogenesis is only licensed once per cell cycle – preventing deviations in centriole number.

At the beginning of the centriole cycle (G1 phase), each cell contains a single centrosome which contains two disengaged centrioles connected by a loose tether at their proximal ends (the G1-G2 tether) (Figure 12A) (Piel et al., 2000; Nigg and Stearns, 2011). These centrioles replicate semi-conservatively during S-phase. The resident centriole, called the mother, assembles a single daughter centriole on its lateral side (Figure 12A) (Kleylein-Sohn et al., 2007; Nigg and Stearns, 2011). During S-phase the cell now contains two engaged centriole pairs encased in PCM, making four centrioles in total (Nigg and Stearns, 2011). The engaged centriole pairs recruit their own PCM during G2 and the procentriole elongates reaching the size of the mother organelle (Figure 12A) (Nigg and Stearns, 2011). The G1-G2 tether connecting the proximal ends of the original resident centrioles is severed during the later stages of G2 in preparation for mitosis (Nigg and Stearns, 2011). This allows the engaged centriole pairs to function as individual centrosomes. Upon passage into mitosis the cell now contains two functional centrosomes, each containing two engaged centrioles (Figure 12A) (Nigg and Stearns, 2011). Given this life cycle, there is a maximum of 4 centrioles per cell at any given time. The centrioles can be visualised by IF in cell lines by staining appropriate centriolar markers. Centrin is a commonly used marker of the centrioles as it is found integrated within the distal lumen of the barrel structure (Paoletti et al., 1996) (Figure 12B). Pericentrin is found localised to the PCM where it functions as a scaffolding protein (Delaval and Doxsey, 2010). It can anchor various proteins to the centrosome to aid centrosome functioning (Delaval and Doxsey, 2010). Therefore pericentrin is a commonly used marker of the outer centrosome structure (Figure 12B).
The U2OS human tumour cell line has a high proportion of cells with normal centriole numbers (Kwon et al., 2008). Therefore using centrin as a marker of the centrioles and pericentrin as a marker of the PCM it is possible to visualise the centrioles by IF and predict cell cycle stage based on centriole:centrosome ratio in untreated U2OS tumour cells (Figure 13).

**Figure 12.** The centrioles and the centriole cycle. A) Schematic of the centriole cycle. B) Diagrammatic representation of the centriole structure with localisation of centrin and pericentrin.
Figure 13. The centriole cycle in U2OS. Centrioles were visualised by IF using centrin as a marker of the centrioles and pericentrin as a marker of the PCM. Images are annotated with prediction of cell cycle phase based on centriole:centrosome ratio. Zoomed images are representative of boxed regions; scale bar=10µm.
In normal cells the centriole cycle is tightly regulated to prevent deviation in centriole number. However, centrosome amplification is increasingly common in human tumour cells. A significant proportion of solid tumours show an increase in centrosome number (Chan, 2011). It is thought that supernumerary centrosomes are beneficial to tumour cells as they can promote chromosomal instability (CIN) and tolerable levels of aneuploidy via the mechanism of centrosome clustering (Basto et al., 2008; Ganem et al., 2009; Kwon et al., 2008). This has been suggested as a potential mechanism of multi-drug resistance in human tumour cells as CIN promotes tumour heterogeneity (Lee et al., 2011). Therefore it is essential to understand the mechanisms by which the centriole cycle becomes disrupted in human tumour cells.

### 3.1.2 Tiam1 depletion increases centrin signal at the centrioles

Several proteins have been implicated in the regulation of the centriole cycle (Brownlee and Rogers, 2013). To investigate the role of Tiam1 in the regulation of centriole number, human tumour cell lines of varying tissue origin were selected based on the criteria that they express Tiam1 and overall, have a normal number of centrioles. Given this, three human tumour cell lines were selected; U2OS (osteosarcoma), HCT116 (colorectal cancer) and MCF7 (breast cancer) (Kwon et al., 2008). To determine whether Tiam1 has a role in regulating centriole number, Tiam1 was transiently depleted by the following siRNA; Tiam1 #1 siRNA. This siRNA was used in all subsequent knockdown experiments unless stated otherwise. This siRNA has been used frequently in our laboratory and has no documented off target effects (Whalley et al., 2015; Woodcock et al., 2010; Vaughan et al., 2015). However to be fully confident that a phenotype observed as a result of siRNA knockdown is a direct result of down-regulation of a specific target protein, the phenotype should be rescued using an RNAi resistant form of the target protein.

Firstly the effect of Tiam1 depletion on centriole number was investigated in U2OS cells. Tiam1 was transiently depleted for 3 days using siRNA. Loss of Tiam1 protein expression was determined by western blot (Figure 14A). Centriole number was assessed by IF using centrin as a marker of the centrioles and pericentrin as a marker of the PCM (Figure 14B). The combined use of a centriole marker and a centrosome marker is essential to be able to confidently identify centrioles that are part of a centrosome.
Centrioles that are associated with a centrosome are more likely to represent functional centrioles. Furthermore the use of both a centriole and centrosome marker can allow the prediction of cell cycle stage.

As discussed, centriole and centrosome number is tightly regulated in normal cells and each cell cycle stage is associated with a distinct number of centrioles and centrosomes. However cell cycle stage is difficult to ascertain in cells with an aberrant centriole/centrosome number as the distinct association between centriole number and cell cycle phase is lost. Therefore the only reliable indicator of cell cycle stage given this combination of markers is the appearance of the nucleus and the DNA, which is only noticeably distinct during mitosis. Given this, any tumour cell with aberrant centriole number will only be categorised as an ‘interphase’ or ‘mitotic’ cell. Centriole number was quantified in U2OS tumour cells from IF images. Aberrant centriole number was categorised as a cell containing ≥5 centrin signals (i.e. anything more than the maximum 4 normal centrioles as described above). Following Tiam1 depletion aberrant centriole structures were located clustered within regions saturated with pericentrin signal, suggesting they are associated with the centrosome. Aberrant centrioles were only quantified if they were associated with a region positive for pericentrin. Given this, following Tiam1 knockdown there is a significant increase in the number of U2OS tumour cells with aberrant centriole number (p=0.0076; ≥145 cells were quantified per experimental replicate; N=3) (**Figure 14C; 14B**). This data suggests that Tiam1 has a regulatory role in the centriole cycle in U2OS tumour cells.
Figure 14. See figure legend p78.
To determine whether this was a cell line specific role of Tiam1, the effect of Tiam1 knockdown was investigated in two additional cell lines, HCT116 and MCF7. Both cell lines express Tiam1 and have a high proportion of cells with a normal number of centrioles (Kwon et al., 2008). Tiam1 was transiently depleted for 3 days by siRNA and protein levels were determined by western blot (Figure 15A). Centriole number was assessed by IF using centrin and pericentrin to mark both the centrioles and the centrosome(s) (as discussed above) (Figure 15B). Cells with aberrant centrioles were classified as those containing ≥5 centrin signals with the additional requirement that signals must be located within a region saturated with pericentrin.

Figure 14. Tiam1 knockdown increases centrin number in U2OS human tumour cell lines. A) Tiam1 was transiently knocked down by siRNA for 3 days in U2OS tumour cell lines. Tiam1 protein levels were detected by western blot using β Actin as a loading control. B) Centrioles were visualised in control (NT siRNA) and Tiam1 knockdown cells by IF using centrin as a marker of the centrioles and pericentrin as a marker of the PCM. Images are annotated with prediction of cell cycle phase based on centriole:centrosome ratio. Zoomed images are representative of boxed regions; scale bar=10µm. C) Centriole number was quantified from an asynchronous population of cells following Tiam1 knockdown. Cells containing ≥5 centrin signals localised with pericentrin were classed as aberrant. ≥145 cells were quantified per experimental replicate. P<0.01(**); bars depict standard error; N=3.

To determine whether this was a cell line specific role of Tiam1, the effect of Tiam1 knockdown was investigated in two additional cell lines, HCT116 and MCF7. Both cell lines express Tiam1 and have a high proportion of cells with a normal number of centrioles (Kwon et al., 2008). Tiam1 was transiently depleted for 3 days by siRNA and protein levels were determined by western blot (Figure 15A). Centriole number was assessed by IF using centrin and pericentrin to mark both the centrioles and the centrosome(s) (as discussed above) (Figure 15B). Cells with aberrant centrioles were classified as those containing ≥5 centrin signals with the additional requirement that signals must be located within a region saturated with pericentrin.
Given these criteria, following Tiam1 knockdown there is a significant increase in the proportion of cells with aberrant centriole number in HCT116 and MCF7 human tumour cell lines (HCT116 p=0.034; ≥145 cells were quantified per experimental replicate; N=3; MCF7 p=0.0369; ≥145 cells were quantified per experimental replicate; N=3) (Figure 15C; 15B). Taken together this data strongly suggests that Tiam1 has a regulatory role in the centriole cycle. As loss of the protein is sufficient to induce the assembly of multiple aberrant centriole structures, this suggests that Tiam1 has a negative effect on centriole duplication. Furthermore as this phenotype is observed in several cell lines derived from varying tissues of origin (U2OS: osteosarcoma, HCT116; colorectal cancer, MCF7; breast cancer), this suggests that this is a general role of Tiam1 and not a phenotype which is cell line specific.

From this point we chose to focus our attention on the U2OS human tumour cell line. This cell line provided a significant increase in the assembly of aberrant centrioles following Tiam1 knockdown. Additionally U2OS cells are large and flat, making them ideal for IF experiments. Furthermore U2OS cells do not grow in clusters – a phenotype observed in both HCT116 and MCF7 - making it easier to accurately quantify centriole number.
Figure 15. See figure legend p81.
Figure 15. Tiam1 knockdown increases centrin number in HCT116 and MCF7 human tumour cell lines. A) Tiam1 was transiently knocked down by siRNA for 3 days in HCT116 and MCF7 tumour cell lines. Tiam1 protein levels were detected by western blot using β Actin as a loading control. B) Centrioles were visualised in control (NT siRNA) and Tiam1 knockdown cells by IF using centrin as a marker of the centrioles and pericentrin as a marker of the PCM. Images are annotated with prediction of cell cycle phase based on centriole:centrosome ratio. Zoomed images are representative of boxed regions; scale bar=10µm. C) Centriole number was quantified from an asynchronous population of cells following Tiam1 knockdown. Cells containing ≥5 centrin signals localised with pericentrin were classed as aberrant. ≥145 cells were quantified per experimental replicate. P<0.05(*); bars depict standard error; N=3.
3.1.3 Tiam1 depletion increases Cp110 signal at the centrioles

The data presented in Section 3.1.2 shows that Tiam1 depletion is sufficient to cause a significant increase in the number of aberrant centrioles visualised in several human tumour cell lines. However this observation has been made using centrin as a marker of the centrioles. Centrin is a commonly used marker of the centrioles as it is found stably integrated within the barrel structure of the centriole (Figure 12B) (Paoletti et al., 1996). However several proteins are fundamental for centriole biogenesis. Experiments in the model system C. elegans have identified 5 core centriolar proteins which are essential for centriole biogenesis (Delattre et al., 2006; Gönczy, 2012; Pelletier, 2006). Functional homologues of these proteins have been identified in other systems (Azimzadeh and Marshall, 2010; Gönczy, 2012; Hodges et al., 2010). Therefore to determine whether aberrant centriole structures in Tiam1 knockdown cells represent fully assembled centrioles further investigation is required. Cp110 is an additional component of the centriole. It is important for centriole biogenesis and functions as a capping protein to regulate the length of the centriole barrel (Kleylein-Sohn et al., 2007; Schmidt et al., 2009) (Figure 16A). Cp110 is closely associated with centrin which is located within the distal lumen of the centriole (Paoletti et al., 1996; Tsang et al., 2006). Figure 16B shows where Cp110 is located on the centriole throughout the cell cycle.
To determine whether Tiam1 depletion causes an increase in Cp110 signal at the centrioles, Tiam1 was transiently depleted for 3 days using siRNA in U2OS cells. Tiam1 protein levels were determined by western blot (Figure 17A). As mentioned above, each centriole is associated with its own Cp110 cap, therefore a maximum of four Cp110 signals can be seen in any cell. Cp110 signal was assessed by IF using centrin as an additional marker of the centrioles (Figure 17B).

Figure 16. Cp110 is localised at the distal end of the centriole. A) Diagrammatic representation of the centriole structure and localisation of Cp110 and centrin. B) Schematic of the centriole cycle and associated Cp110 signal.
Figure 17. See figure legend p85.
U2OS cells containing ≥5 Cp110 signals across an asynchronous cell population were classed as aberrant. Following Tiam1 knockdown there is an increase in the number of U2OS cells with aberrant Cp110 signal (p=0.0646; ≥43 cells were quantified per experimental replicate; N=3) (Figure 17C; 17B). This suggests that supernumerary centrin signals assembled following loss of Tiam1 recruit additional centriolar components. As there was some variation between experimental replicates the overall result of this experiment was not significant. This experiment needs to be repeated to determine whether loss of Tiam1 causes a significant increase in Cp110 signal – particularly as the p value is close to providing a significant result (p=0.0646). Despite this, taken together this preliminary data supports the hypothesis that additional centrin signals formed as a result of Tiam1 depletion represent fully developed centrioles.
3.1.4 Cp110 associates with additional centrin structures in Tiam1 knockdown cells

Gated STED (gSTED) microscopy has revolutionised our understanding of cellular biology. Its creators Stephan W. Hell, Eric Betzig and William E. Moerner recently won the Nobel Prize for chemistry for the development of the gSTED system and super resolution microscopy (2014). With the development of gSTED it is now possible to visualise the nanostructure of cellular organelles, with an improved lateral resolution of ~30nm (Hell and Wichmann, 1994). Standard confocal microscopy can only achieve a lateral resolution of ~200nm (Huang et al., 2009). This technology has been particularly ground breaking in the field of centriole biology. Due to their size (~ 450nm length, ~ 250nm width; Gönczy, 2012) and organisation (mother and daughter are often engaged in an L-shape conformation; Gönczy, 2012), it can be challenging to determine centriole number using standard confocal microscopy. Due to the range in lateral resolution a single centriole signal obtained from a confocal system may represent two centrioles in close proximity. Figure 18 compares images taken on a standard confocal microscope and a gSTED system – images show supernumerary centrioles aberrant centriole structures found in Tiam1 knockdown cells. Using the gSTED system, centrin is clearly identifiable as multiple discrete units and number of centrin signals can be confidently quantified. However using a confocal system centrin appears clustered. These clustered regions are likely to contain multiple centrin signals but appear as one large signal due to the range of lateral resolution. This makes it challenging to fully quantify centriole number. Therefore the use of gSTED microscopy is likely to further strengthen the aberrant centriole structure phenotype found in Tiam1 knockdown cells – this may be under-represented by conventional confocal microscopy.
To further elucidate the relationship between aberrant centrin and Cp110 signals visualised following loss of Tiam1, gSTED microscopy was used to examine the localisation of each centriolar protein in U2OS human tumour cells. Tiam1 was knocked down for 3 days by siRNA and protein levels were detected by western blot (Figure 19A). Cells were selected for the analysis based on centriole number - U2OS cells with a normal number of centrioles (2-4 centriole signals) were used as a control (NT siRNA) whilst Tiam1 knockdown cells with aberrant centrioles (≥5 centriole structures) were selected for analysis (10 cells were analysed per experimental replicate, N=2) (Figure 19B).

Figure 18. Comparison of centrioles visualised by standard confocal microscopy and gSTED microscopy. Images compare aberrant centriole structures visualised by confocal microscopy and gSTED microscopy in Tiam1 knockdown cells. Centrin was used as a marker of the centrioles and pericentrin was used as a marker of the PCM. Scale bar=1µm.
Figure 19. Aberrant centriole structures have a Cp110 cap. A) Tiam1 was transiently depleted for 3 days by siRNA in U2OS tumour cells. Protein levels were detected by western blot using β Actin as a loading control. B) gSTED microscopy was used to visualise Cp110 and centrin localisation in control (NT siRNA) and Tiam1 knockdown cells. Images are annotated with a schematic of predicted Cp110:centrin relationship. 10 cells were analysed per experimental replicate. Images are representative of N=2. Scale bar=1µm.
The increased resolution of the gSTED system allows deeper understanding of the relationship between centrin and Cp110 following Tiam1 knockdown. Each aberrant centrin signal is associated with a Cp110 signal and it is possible to see Cp110 localised to the edges of each centrin signal – resembling the capping nature of the Cp110 protein. It was also easier to identify individual centrin structures as each signal is resolved to a discrete dot. Interestingly, following loss of Tiam1 aberrant centrin structures appear in pairs. This may represent multiple engaged mother-daughter centriole pairs as shown in schematics in Figure 19B. Taken together this data further suggests that aberrant centriole structures assembled as a result of Tiam1 depletion recruit the necessary components to construct a fully developed centriole organelle.

3.2 Supernumerary centrioles in Tiam1 depleted cells are functional and have the potential to promote chromosomal instability

3.2.1 Introduction

Centrosome amplification is a common feature of tumour cells – tumour cells are thought to benefit from supernumerary centrosomes due to a mechanism called ‘centrosome clustering’ (Basto et al., 2008; Ganem et al., 2009; Kwon et al., 2008; Lee et al., 2011). Centrosome clustering allows the tumour cell to maintain a bipolar spindle during mitosis, however as supernumerary centrosomes cluster – several transient multipolar spindles are assembled (Ganem et al., 2009). These transient intermediates promote inappropriate MT-kinetochore attachments – primarily merotelic attachments (Ganem et al., 2009; Thompson et al., 2010). Merotelic attachments are undetected by the SAC and if left unrepaired promote lagging chromosomes at anaphase – a major contributor to CIN (Gregan et al., 2011; Thompson and Compton, 2008; Thompson et al., 2010). However, this is only applicable if aberrant centriole structures are functional, can nucleate MTs and contribute to spindle assembly. To determine whether aberrant centriole structures in Tiam1 knockdown cells are capable of promoting CIN – by facilitating the assembly of transient multipolar intermediates and lagging chromosomes at anaphase – the functionality of aberrant centriole structures in Tiam1 knockdown cells must be investigated. Aberrant centriole structures assembled as a result of Tiam1 knockdown must be capable of nucleating MTs and have the capacity to contribute to spindle assembly.
3.2.2 Aberrant centrioles in Tiam1 knockdown cells persist following depolymerisation of the microtubule network

The centrosome is the MTOC of the cell – its primary function is to nucleate MTs (Azimzadeh and Bornens, 2007). Within the core of the centrosome are the centrioles, and the centriole cycle dictates the formation of the centrosome (Nigg and Stearns, 2011). Centriolar satellites are dense, fibrous structures found localised in close proximity to the centrosome (Bärenz et al., 2011). They shuttle proteins such as centrin, pericentrin and ninein to the centrosome in a dynein dependant manner but themselves do not represent bona fide centriole organelles – they do not function as the core of the MTOC (Balczon et al., 1999; Dammerman and Merdes, 2002; Kubo et al., 1999; Löffler et al., 2013). Excessive centriolar satellite formation has been shown to precede centrosome amplification (Löffler et al., 2013). Therefore supernumerary centrioles in Tiam1 knockdown cells may represent centriolar satellites – Tiam1 knockdown may increase the shuttling of centrin to the centrioles without representing bona fide centriole amplification. Centriolar satellites are dependent on the MT network and it has been show that depolymerisation of this network is sufficient to scatter centriolar satellites throughout the cytoplasm (Kubo et al., 1999; Tsang et al., 2009). Therefore centriolar satellite localisation at the centrosome periphery is compromised by cold treatment.

To determine whether supernumerary centrioles in Tiam1 knockdown cells are centriolar satellites and do not form the core of the MTOC, Tiam1 was transiently depleted for 3 days by siRNA (Figure 20A). The cytoplasmic MT network was depolymerised in control (NT siRNA) and Tiam1 knockdown cells by treatment on ice for 30 minutes. Centriole number was assessed in the absence of cytoplasmic MTs by IF using centrin as a marker of the centrioles, pericentrin as a marker of PCM and α tubulin as a marker of the cytoplasmic MT network (Figure 20B). Aberrant centrioles (≥5 centrin signals) were only quantified if they remained associated with a region positive for pericentrin and were not scattered throughout the cytoplasm. Following depolymerisation of the cytoplasmic MT network there is a significant increase in the number of cells with aberrant centriole number (p=0.0112; ≥70 cells were quantified per experimental replicate; N=5) (Figure 20C; 20B). Notably aberrant centrioles remained clustered to regions that were associated with pericentrin. This data suggests that supernumerary centrioles in Tiam1 knockdown cells represent stable organelles that function as part of the centrosome.
Figure 20. See figure legend p92.
3.2.3 Aberrant centrioles in Tiam1 knockdown U2OS cells can nucleate microtubules

Supernumerary centrioles in Tiam1 knockdown cells appear resistant to de-polymerisation of the cytoplasmic MT network, suggesting they represent stable centrosome components. The centrioles form the core of the MTOC – however the ability to nucleate MTs is regulated by the PCM components (Moritz et al., 2000). Gamma tubulin ring complexes (γTuRCs) are embedded within the PCM and act as docking sites for MT nucleation (Moritz et al., 2000). Although the centriole remains crucial in this process as it recruits PCM, the centriole alone is not sufficient to function as an MTOC (Gould and Borisy, 1977). Therefore to potentially promote CIN by the assembly of transient multipolar spindle intermediates, supernumerary centrioles in Tiam1 knockdown cells must function as an MTOC and nucleate MTs.

To determine whether aberrant centrioles in Tiam1 knockdown cells are capable of recruiting PCM and nucleating MTs, the ability of supernumerary centrioles to assemble MT asters was assessed in U2OS tumour cells. Tiam1 was transiently depleted for 3 days using siRNA and protein levels were detected by western blot (Figure 21A). Following incubation on ice for 30 minutes, the cytoplasmic MT network was depolymerised and there are no MTs visible by IF in U2OS tumour cells (data shown in Figure 20B). Following incubation on ice, control (NT siRNA) and Tiam1 knockdown cells were transferred to pre-heated media for 20 seconds to encourage a short burst of microtubule nucleation. Microtubule aster formation was assessed by high resolution gSTED microscopy. As discussed in Section 3.1.4, the gSTED system greatly improves the quality of images obtained by IF.
Figure 21. Aberrant centrioles are associated with localised regions of microtubule nucleation. A) Tiam1 was transiently depleted for 3 days using siRNA in U2OS tumour cells. Protein levels were detected by western blot with β Actin used as a loading control. B) Sites of MT nucleation were visualised in control (NT siRNA) and Tiam1 knockdown cells by gSTED microscopy, using α Tubulin as a marker of the MTs, centrin as a marker of the centrioles and pericentrin as a marker of the PCM. Annotated regions show localisation of centrioles in comparison to regions of MT nucleation. 10 cells were visualised per experimental replicate. Images are representative of N=2. Scale bar=1µm.
It is clear that aberrant centrioles in Tiam1 knockdown cells are associated with localised regions of MT nucleation and are clustered to areas saturated with pericentrin (10 cells were analysed per experimental replicate, N=2) (Figure 21B). This data strongly suggests that aberrant centriole structures can recruit PCM and are capable of nucleating microtubules – they can function as an MTOC and have the potential to contribute to spindle assembly during mitosis.

3.2.4 Supernumerary centrioles contribute to spindle assembly

The data presented in Section 3.2.3 suggests that supernumerary centrioles can function as a MTOC and can potentially contribute to spindle assembly during mitosis. However it has been hypothesised that supernumerary centrosomes can be deactivated prior to mitosis. Inactive centrosomes do not associate with MTs and do not localise to the spindle poles (Basto et al., 2008). This prevents supernumerary centrosomes interfering with bipolar spindle assembly, consequently preventing lagging chromosomes at anaphase and CIN.

To ensure that aberrant centrioles in Tiam1 knockdown cells are capable of contributing to spindle assembly, centriole number was assessed in mitotic cells exclusively. Two independent siRNA oligos were used in this experiment, Tiam1 #1 siRNA (discussed in Section 3.1.2) and Tiam1 #2 siRNA (Figure 22).
Tiam1 was transiently depleted for 3 days and protein levels were detected by western blot (Figure 23A). Mitotic cells were assessed exclusively in this experiment. A normal mitotic cell should contain a single centrosome containing 2 centrioles at each spindle pole (4 centrioles; 2 centrosomes in total). Centriole number was assessed by IF using centrin as a marker of centrioles and pericentrin as a marker of the PCM (Figure 23B). Aberrant centriole number was classed as a mitotic cell containing ≥5 centrin signals localised within regions positive for pericentrin. Following Tiam1 knockdown there is a significant increase in the number of mitotic cells with supernumerary centrioles associated with the spindle poles (Tiam1 #1 siRNA: p=0.0046; 100 mitotic cells quantified per experimental replicate; N=3, Tiam1 #2 siRNA: p=0.0024; ≥45 mitotic cells quantified per experimental replicate; N=3) (Figure 23C; 23B). This result was replicated using two independent siRNA oligos against the Tiam1 mRNA. This data shows that aberrant centrioles are associated with pericentrin at the spindle poles and are not de-activated prior to mitosis. Therefore this data strongly suggests that aberrant centrioles actively contribute to spindle assembly and have the potential to promote lagging chromosomes at anaphase – a major contributor of CIN.
Figure 23. See figure legend p97.
Figure 23. Tiam1 knockdown increases the number of aberrant centriole structures localised at the spindle poles during mitosis. A) Tiam1 was transiently depleted for 3 days in U2OS tumour cells using two independent siRNAs; Tiam1 #1 siRNA (used in previous experiments) and Tiam1 #2 siRNA. Protein levels were detected by western blot using β Actin as a loading control. B) Centrioles were visualised in mitotic cells by IF using centrin as a marker of the centrioles, pericentrin as a marker of the PCM and DAPI to confirm cell cycle phase. Scale bar=10µm. C) Centriole number was quantified in mitotic cells exclusively. Control (NT siRNA) and Tiam1 knockdown cells containing ≥5 centrin signals were classed as aberrant. ≥45 mitotic cells were quantified per experimental replicate. P<0.01(**); bars depict standard error; N=3.
3.2.5 Loss of Tiam1 correlates with an increased frequency of lagging chromosomes at anaphase

The data presented in Section 3.2.4 suggests that aberrant centrioles in Tiam1 knockdown cells actively contribute to spindle assembly. Functional supernumerary centrosomes have been shown to promote lagging chromosomes at anaphase – a consequence of centrosome clustering promoting inappropriate MT-kinetochore attachments at the chromatids (Thompson et al., 2010). Lagging chromosomes are considered to be a major contributor to CIN (Thompson and Compton, 2008; Thompson et al., 2010).

To determine whether aberrant centrioles in Tiam1 knockdown cells have the potential to promote CIN, Tiam1 was transiently depleted for 3 days by siRNA in U2OS tumour cells (Figure 24A). Lagging chromosomes were visualised by IF and categorised as chromatids that had not segregated with the main body of chromosomes during anaphase. DAPI was used to detect chromatids, centrin and pericentrin were used to highlight the components of the centrosome, and α tubulin was used to highlight the spindle MTs (Figure 24B). The frequency of lagging chromosomes at anaphase was quantified in control (NT siRNA) and Tiam1 knockdown cells. Following Tiam1 knockdown there is a significant increase in the frequency of lagging chromosomes at anaphase (p=0.006911; ≥100 anaphase cells quantified per experimental replicate; N=3) (Figure 24C; 24B). Furthermore, Tiam1 knockdown also caused a significant increase in the number of anaphase cells with supernumerary centrioles at the spindle poles (p=0.003056; ≥100 anaphase cells quantified per experimental replicate; N=3) (Figure 24D). Taken together this data suggests that aberrant centrioles may play a direct role in promoting chromosome mis-segregation. Furthermore this data provides additional evidence that aberrant centrioles in Tiam1 depleted cells are functional – they remain at the spindle poles during anaphase and are associated with a phenotypic output (increase in lagging chromosomes). This strongly suggests that aberrant centrioles can interfere with bipolar spindle assembly and therefore have the ability to contribute to CIN.

Data presented in Section 3.2.5 was kindly provided by Helen Whalley, a previous post doc in the laboratory.
Figure 24. See figure legend p100.
Figure 24. Tiam1 knockdown results in an increase in frequency of lagging chromosomes at anaphase. A) Tiam1 was knocked down by siRNA for 3 days in U2OS tumour cells. Protein levels were detected by western blot using β Actin as a loading control. B) Lagging chromosomes were visualised by IF using DAPI as a marker of DNA. α Tubulin was used as a marker of MTs with centrin and pericentrin used to mark the centrioles and PCM. Arrow points to a lagging chromosome. Scale bar=10µm. C) The frequency of lagging chromosomes at anaphase was quantified in control (NT siRNA) and Tiam1 knockdown cells. Chromosomes that failed to separate with the main body of DNA during anaphase were classed as lagging chromosomes. ≥100 anaphase cells were quantified per experimental replicate. P<0.01(**); bars depict standard error; N=3. D) Centrin signal was also quantified in control (NT siRNA) and Tiam1 knockdown anaphase cells. Cells containing ≥5 centrin signals were classed as aberrant. ≥100 anaphase cells were quantified per experimental replicate. P<0.01(**); bars depict standard error; N=3.

The data in this figure was kindly provided by Helen Whalley, a previous post doc in the laboratory.
3.3 Discussion and Future Plans

Centrosome amplification is rarely seen in normal cells because the centriole cycle is highly regulated to prevent deviations in centriole number (Brownlee and Rogers, 2013; Nigg, 2002; Pihan et al., 1998; Sato et al., 1999). However centrosome amplification occurs frequently in human tumours with deviations in centrosome number found in tumour cells derived from various tissues (Chan, 2011). It has been hypothesised that supernumerary centrosomes are beneficial to tumour development as centrosome clustering can promote CIN, a mechanism that enhances tumour heterogeneity and potentially contributes to multi-drug resistance (Ganem et al., 2009; Lee et al., 2011; Thompson and Compton, 2008). This makes centrosome amplification an attractive target for drug development. However to fully understand how centrosome amplification is achieved in tumour cells it is crucial to understand the mechanisms that regulate the centriole cycle. We have identified Tiam1 as a novel regulator of the centriole cycle.

3.3.1 Aberrant centriole structures represent fully assembled centriole organelles

Tiam1 knockdown causes the assembly of aberrant centriole structures in human tumour cell lines. This was observed in tumour cells derived from various tissue origins; bone (U2OS), colon (HCT116) and breast (MCF7), suggesting this is a general role of Tiam1 and not a cell line specific effect. This strongly suggests that Tiam1 has a regulatory role in the centriole cycle – specifically regulating centriole number. However the effect of Tiam1 knockdown was mainly assessed using a single siRNA – Tiam1 #1 siRNA. Experiments in mitotic cells were repeated with two siRNAs and both induced the formation of aberrant centriole structures – indicating this is not an off target effect of Tiam1 #1 siRNA. Furthermore this oligo has been used repeatedly within the laboratory and has shown no off target effects (Whalley et al., 2015; Woodcock et al., 2010; Vaughan et al., 2015). However more experiments should be repeated with a second oligo to confirm that this is a true phenotype of Tiam1. To further validate that Tiam1 knockdown causes the assembly of aberrant centriole structures an RNAi resistant Tiam1 should be re-expressed in to cells treated with Tiam1 #1 siRNA. From the data presented in this chapter, this should be sufficient to rescue the aberrant centriole phenotype.
Following Tiam1 knockdown aberrant centrioles were only quantified if they localised to regions positive for pericentrin – a marker of the PCM. This provides preliminary evidence that these structures are associated with a centrosome and are potentially capable of functioning as a MTOC. Centrin was used as a marker to evaluate centriole number. As discussed, this is a commonly used marker of centrioles as it is localised within the distal lumen of the centriole barrel (Paoletti et al., 1996). However, numerous proteins are required to build a functional centriole. There are examples of structures that resemble centrioles (centriolar satellites) which are positive for some centriolar components but are unable to function as a MTOC (Balczon et al., 1999; Dammerman and Merdes, 2002; Kubo et al., 1999; Löffler et al., 2013). Therefore this data alone is not sufficient to conclude whether aberrant centrioles in Tiam1 depleted cells represent fully assembled, functional centrioles. Cp110 is a crucial component of the centrioles. Cp110 has been described as a ‘centriole cap’ that sits on the distal end of the centriole and restricts the length of centriole barrel (Kleylein-Sohn et al., 2007; Schmidt et al., 2009). Following Tiam1 depletion there is an increase in the number of cells with aberrant Cp110 signal. At this stage, the data is not statistically significant due to the variation seen over three experimental replicates. However the calculated p value is close to providing a significant result (p=0.0646). Therefore this experiment should be repeated to finally conclude if Tiam1 knockdown has a significant effect on Cp110 staining. Further investigation of Cp110 using gSTED microscopy highlighted a distinct relationship between aberrant centrin signals and Cp110. It was evident that aberrant centrin signals found in Tiam1 knockdown cells are associated with their own Cp110 signal. With the increased resolution of the gSTED system it was even possible to see the localisation of Cp110 to the ends of the centrin signal, highlighting the ‘capping’ nature of the protein. Cp110 is one of the last proteins to be assembled on the centriole during biogenesis (Kleylein-Sohn et al., 2007). Therefore it is promising that aberrant centriole structures are associated with protein components that function at the end of the centriole biogenesis pathway – this suggests that these structures represent fully assembled centrioles.

Taken together this data suggests that loss of Tiam1 promotes the assembly of complete centriole structures. However to further strengthen this data the signal frequency of additional centriolar components should be quantified.
For example, following Tiam1 knockdown the frequency of SAS-6 signal could be examined. SAS-6 is fundamental for centriole biogenesis, it forms the base of the centriole and is crucial for templating the unique 9-fold symmetry of the barrel (Leidel et al., 2005; Nakazawa et al., 2007). Furthermore SAS-6 is localised to the proximal region of the centriole, whilst Cp110 and centrin are localised at the distal end of the barrel. Combining these three centriolar components could build a ‘3D’ representation of the barrel shaped organelle. Visualising the centrioles in such molecular detail by IF has only become possible with the recent development of super-resolution microscopy (gSTED). Visualisation of the molecular detail of each centriolar component following Tiam1 knockdown may yield further insight in to the mechanism by which loss of Tiam1 results in excessive centriole assembly.

3.3.2 Aberrant centrioles in Tiam1 knockdown cells behave like fully assembled centrioles

This data has shown that loss of Tiam1 promotes the assembly of supernumerary centrioles. It is widely accepted that centrosome amplification is a common feature of tumour cells and supernumerary centrosomes have the potential to promote CIN (Ganem et al., 2009; Thompson and Compton, 2008; Thompson et al., 2010). However, this is only applicable if aberrant centrioles in Tiam1 knockdown cells are functional and can contribute to spindle assembly. Our data has shown that loss of Tiam1 promotes the assembly of supernumerary centrioles which are positive for more than one centriolar component and are localised to regions positive for pericentrin, a marker of the PCM. Therefore as aberrant centrioles are associated with PCM, this suggests they form part of the centrosome. However this does not categorically confirm that additional centrioles in Tiam1 knockdown cells are functional and capable of nucleating MTs. Centriolar satellites are dense, fibrous structures which appear to ‘orbit the centrosome’ (Bärenz et al., 2011). They are crucial for transporting centrosome components along the MT network but themselves do not recruit all the proteins necessary to form a functional centriole and MTOC. However their close proximity to the centrosome can cause them to be mistaken for centrioles. As centriolar satellites rely heavily on the MT network for cargo shuttling to the centrosome, their assembly at the periphery of the centrosome has been shown to be dependent on dynein and the cytoplasmic MT network (Balczon et al., 1999; Kubo et al., 1999).
Loss of this network forces the satellites to scatter throughout the cytoplasm (Dammermann et al., 2002; Kubo et al., 1999; Sedjaï et al., 2010). Furthermore fully assembled centrioles are heavily acetylated and glutamylated which stabilises the centriole structure – ensuring the centriole is not affected by the dynamic nature of the cytoplasmic MTs (Edde et al., 1990; Kochanski and Borisy, 1990; Piperno and Fuller, 1985). Therefore changes to the cytoplasmic MT network will have no effect on *bona fide* centriole assembly. Depolymerisation of the cytoplasmic MT network had no effect on assembly of supernumerary centrioles – centrin signals remained clustered to pericentrin positive regions and were not dispersed throughout the cytoplasm. This suggests that aberrant centrioles represent stable centriole components and are not centriolar satellites. This provides further evidence that loss of Tiam1 promotes the assembly of *bona fide* centrioles that form the core of the centrosome.

To further test our hypothesis that aberrant centrioles behave like functional centrioles, the ability of these structures to recruit PCM and nucleate MTs was assessed. Using gSTED microscopy it was evident that aberrant centrioles are associated with PCM and can nucleate MTs – characteristics displayed by functional MTOC’s. To further strengthen this data, levels of MT nucleation could be assessed using live imaging to determine the overall rates of MT re-growth. If aberrant centrioles in Tiam1 knockdown cells are functional and can facilitate MT nucleation there should be an increase in the amount of MTs nucleated in Tiam1 knockdown cells when compared to control. Additionally aberrant centrioles should be assessed with a γ tubulin marker. γTuRCs are found embedded within the PCM and these structures are crucial for initiating microtubule nucleation (Moritz et al., 2000). If aberrant centrioles can nucleate MTs they should also be associated with γ tubulin.

### 3.3.3 Aberrant centriole structures contribute to spindle assembly and have the potential to promote CIN

Centrosome amplification is thought to promote CIN during the centrosome clustering process (Ganem et al., 2009). As supernumerary centrosomes cluster they form transient multipolar spindles which promote inappropriate MT-kinetochore attachments – resulting in lagging chromosomes and CIN (Ganem et al., 2009; Thompson et al., 2010).
However it has been shown in some cell lines that supernumerary centrosomes can be inactivated prior to mitosis (Basto et al., 2008). This prevents interference with bipolar spindle assembly, eliminating the possibility of CIN via centrosome clustering. However, following Tiam1 knockdown there is still a significant increase in the number of mitotic cells with supernumerary centrioles associated with PCM at the spindle poles. Therefore aberrant centrioles are not deactivated prior to mitosis. Notably this result was replicated using two independent siRNA oligos against Tiam1 – further confirming that depletion of Tiam1 stimulates the assembly of supernumerary centrioles. These structures are retained at the spindle poles, therefore the cell must actively promote the clustering of aberrant centrioles – this action must have some beneficial phenotype to the tumour cell. Retaining aberrant centrioles may be beneficial as they have the potential to promote CIN. In support of this, Tiam1 depletion correlates with a significant increase in the frequency of lagging chromosomes at anaphase – a phenotype that is considered to be a major contributor to CIN (Thompson and Compton, 2008; Thompson et al., 2010). Therefore Tiam1 knockdown is associated with a phenotypic output, further suggesting aberrant centrioles are functional and can alter the dynamics of mitotic spindle assembly. This phenotype was also accompanied with a significant increase in the frequency of aberrant centrioles found at the poles of anaphase cells – supportive of the hypothesis that these structures actively contribute to the lagging chromosome phenotype. It has been shown that loss of Tiam1 induces excessive centrosome separation during mitosis and this phenotype correlates with an increase in chromosome congression errors during metaphase (Whalley et al., 2015; Woodcock et al., 2010). However it is unlikely that chromosome congression errors are sufficient to promote lagging chromosomes at anaphase – particularly as this phenotype is associated with increased time spent in mitosis (Woodcock et al., 2010). This suggests that mitosis is halted until all chromosomes are aligned at the metaphase plate and correctly attached to MTs. However to further test that aberrant centrioles directly cause an increase in lagging chromosome incidence, long term live imaging could be used to track cells with supernumerary centrioles. Lagging chromosomes are a product of centrosome clustering (Ganem et al., 2009). As supernumerary centrioles cluster towards the spindle poles, multiple transient multipolar intermediates are produced (Ganem et al., 2009). This promotes inappropriate MT-kinetochore attachments, resulting in lagging chromosomes at anaphase (Thompson and Compton, 2008).
Cells with aberrant centrioles assembled as a result of loss of Tiam1 should also form transient multipolar spindles as they progress through mitosis. This would further support the hypothesis that aberrant centrioles in Tiam1 depleted cells are functional and are capable of contributing to CIN in human tumour cell lines.

3.3.4 Conclusion

To comprehensively confirm that aberrant centrioles in Tiam1 knockdown cells represent fully functional organelles, electron microscopy should be used to visualise the unique barrel shaped structure of the centriole. This will definitively prove that aberrant centrioles are fully assembled centrioles. However the data presented in this chapter has shown that Tiam1 knockdown induces the formation of aberrant centrioles which are positive for more than one centriole component and are associated with pericentrin. Furthermore these supernumerary centrioles mirror the stable nature of the centriole organelle and are associated with regions of MT nucleation - the principle role of the centriole is to recruit PCM and nucleate MTs. Also, additional centrioles are retained during mitosis as aberrant centrioles can be visualised at the spindle poles. Furthermore, inducing the formation of supernumerary centrioles by knocking down Tiam1 correlates with an increased frequency of lagging chromosomes at anaphase. This is a principle phenotype observed in tumour cells with supernumerary centrosomes (Ganem et al., 2009). Taken together this data strongly suggests that Tiam1 plays a regulatory role in the centriole cycle. Loss of this protein is sufficient to promote the assembly of supernumerary centrioles which have the capacity to interfere with chromosome segregation during mitosis.
Chapter 4. Tiam1 Regulates Centriole Number by Preventing Centriole Re-duplication

4.1 Tiam1 prevents centriole re-duplication

4.1.1 Introduction

Supernumerary centrosomes can be detrimental to normal cells as they promote multipolar spindles during mitosis (Dodson et al., 2004; Ganem et al., 2009). This can lead to cell death by large scale aneuploidy (Ganem et al., 2009). For this reason, the centriole cycle is tightly regulated in normal cells to maintain a stable centriole number. However centrosome amplification is a common feature of human tumours (Chan, 2011). Therefore during conversion of normal cells to malignant cells, key regulatory mechanisms become altered, resulting in centrosome amplification. Whether centrosome amplification is a cause or consequence of malignant progression is very much under debate (Nigg, 2002; Zyss and Gergely, 2009).

The data presented in Chapter 3 strongly suggests that Tiam1 has a regulatory role in the centriole cycle. More specifically, loss of Tiam1 is sufficient to promote the assembly of multiple aberrant centrioles. However the mechanism by which additional centriole structures are produced following depletion of Tiam1 is unknown. From what is already known about the centriole cycle and modes of centrosome amplification, it is thought supernumerary centrioles can arise via three independent pathways. Firstly, defective mitosis can result in supernumerary centrioles, even when the centriole cycle remains functional. Failure to exit mitosis correctly, either by incomplete cytokinesis or mitotic slippage will result in aberrant centriole number (Meraldi et al., 2002). However in this case supernumerary centrioles are a bi-product of faulty mitosis and not a result of de-regulation of the centriole cycle (Meraldi et al., 2002). Secondly, de-regulation of the centriole cycle will promote supernumerary centrioles. The centriole cycle is tightly coupled to the cell cycle - ensuring centriole duplication is initiated once per cell cycle. Uncoupling of these processes can result in centriole amplification (Balczon et al., 1995). Additionally centriole duplication is semi-conservative, where the mother centriole templates the formation of a daughter centriole. The mother centriole is permitted to produce a single daughter per centriole cycle.
Loss of copy number control and the over-production of daughter centrioles within a single cell cycle will promote supernumerary centrioles (Habedanck et al., 2005; Kleylein-Sohn et al., 2007).

Tiam1 has been implicated in the regulation of spindle assembly. Tiam1 counteracts the action of Eg5 in early mitosis to promote centrosome separation, a process which is fundamental for bipolar spindle assembly (Woodcock et al., 2010; Whalley et al., 2010). Loss of Tiam1 promotes increased centrosome separation whilst inhibition of Eg5 prevents centrosome separation resulting in monopolar spindle formation (Blangy et al., 1995; Sawin et al., 1992; Whalley et al., 2015; Woodcock et al., 2010). Combined repression of Tiam1 and Eg5 prevents any interference with bipolar spindle assembly, rescuing defective mitosis (Woodcock et al., 2010). Therefore Tiam1 is implicated indirectly in the progression of mitosis. Supernumerary centrioles were not documented in the findings of either Whalley at al., 2015 or Woodcock et al., 2010. However evidence presented in Chapter 3 shows that loss of Tiam1 in the following human tumour cell lines; U2OS, HCT116 and MCF7 is sufficient to promote the assembly of supernumerary centrioles. Therefore Tiam1 must also be involved in regulating one of the following processes; mitotic exit, coupling of the centriole cycle to the cell cycle or daughter copy number control.

4.1.2 Tiam1 knockdown does not prevent mitotic exit

Initially the role of Tiam1 in mitotic exit was investigated. Supernumerary centrioles can be the bi-product of failure to exit mitosis, either by cytokinesis failure or mitotic slippage (Godinho and Pellman, 2014; Meraldi et al., 2002). Tiam1 was knocked down for 3 days by siRNA in U2OS tumour cells and protein levels were detected by western blot (Figure 25A). Control (NT siRNA) and Tiam1 knockdown cells were stained with propidium iodide to highlight DNA and flow cytometry was used to determine cell cycle profiles. Flojo software was used to determine the proportion of cells found in each cell cycle phase (Figure 25B). The average frequency of cells found in each cell cycle phase in control (NT siRNA) and Tiam1 knockdown U2OS was calculated using Flojo data obtained over 3 independent experiments. Following Tiam1 knockdown there was no significant change in cell cycle profiles when compared to control, (≥1x10^4 cells were analysed per experimental replicate; N=3 (Figure 25C; 25B).
Figure 25. See figure legend p110.
Notably there was no increase in the frequency of cells in G2/M-phase of the cell cycle following Tiam1 knockdown – no significant tetraploid population was observed. Furthermore there were no cells identified as being highly polyploid. This would have presented as multiple peaks higher than 400 FL2-A on the histogram data – this would indicate cells with a higher DNA content than 2N (Figure 25B). Taken together this data suggests - in this biological setting - that loss of Tiam1 has no role in regulating exit from mitosis. Therefore the assembly of aberrant centrioles following Tiam1 loss is not a result of faulty cytokinesis or mitotic slippage.

4.1.3 Tiam1 over-expression partially rescues centriole amplification caused as a result of hydroxyurea treatment

The data presented in Section 4.1.2 suggests that loss of Tiam1 has no effect on mitotic exit in U2OS tumour cells, suggesting that Tiam1 does not contribute to the licensing of mitotic exit. We therefore tested whether loss of Tiam1 results in de-regulation of the centriole cycle. As discussed, coupling of the centriole cycle and the cell cycle is crucial to maintain a normal centriole number. In some cell lines, deviations in the cell cycle can result in centriole amplification. For example, extended S-phase induced by hydroxyurea can promote centriole amplification (Balczon et al., 1995; Tsang et al., 2009). Similarly, extending the time spent in G2 can also promote centriole amplification (Dodson et al., 2004; Lončarek et al., 2010).
Alterations within the cell cycle which allow changes in the temporal expression of key proteins involved in centriole biogenesis is sufficient to promote centriole amplification. Two groups of proteins have been identified as key regulators of the centriole cycle; proteins that positively influence centriole biogenesis and proteins that have a negative effect on centriole assembly (Brownlee and Rogers, 2013; Balestra et al., 2013). The mechanism by which proteins restrict centriole duplication remains unclear. Some proteins have been shown to down-regulate proteins involved in centriole biogenesis (Brownlee et al., 2011), whilst others have been shown to maintain the physical interaction between the centriole pairs (the S-M linker) (Lončarek et al., 2010).

Hydroxyurea artificially induces centriole amplification in some cell lines. It inhibits ribonucleotide reductase, reducing the levels of dNTPs available during S-phase - blocking DNA synthesis (Koç et al., 2004). However it has been shown that hydroxyurea does not completely abolish the dNTP pool, therefore it has been suggested that DNA replication does not completely halt, it progresses at a much reduced rate (Alvino et al., 2007). Treatment by hydroxyurea in some cell lines allows the continual expression of proteins which are crucial for licensing centriole duplication – resulting in centriole amplification (Balczon et al., 1995; Tsang et al., 2009). By proof of principle, over-expression of proteins that have a negative effect on centriole assembly have been shown to sufficiently rescue centriole amplification caused as a result of defects in the cell cycle (Ferguson and Maller, 2008; Tsang et al., 2009).

To determine whether Tiam1 is capable of restraining excessive centriole duplication, Tiam1 was stably over-expressed in U2OS tumour cells. A construct containing GFP-tagged Tiam1 was transfected into U2OS cells and exogenous expression was maintained by culturing the cells in selection (G418). As a control, U2OS cells exogenously expressing GFP were made following the same method. Exogenous Tiam1 was detected by western blot (Figure 26A). As the levels of exogenous Tiam1 detected by western blot were low, Tiam1-GFP expression was examined by IF using an antibody against the GFP tag. Cells expressing high levels of Tiam1-GFP could be detected by IF (Figure 26B). Control (GFP-only) and Tiam1-GFP over-expressing cells were treated with hydroxyurea for 4 days to induce centriole amplification and GFP positive cells containing ≥5 centrioles were classed as aberrant. Significantly, cells which over-express Tiam1 had a reduced level of centriole amplification when compared to control (p=0.0022; 50 GFP positive cells were quantified per experimental replicate; N=3) (Figure 26C).
Centriole number was also quantified in control (GFP only) and Tiam1 over-expressing cells in the absence of hydroxyurea. GFP positive cells containing ≥5 centrioles were classed as aberrant. Notably, over-expression of Tiam1 in the absence of hydroxyurea had no effect on normal centriole duplication (p=0.5614; 50 GFP positive cells were quantified per experimental replicate; N=3) (Figure 26D). This data strongly suggests that Tiam1 has a negative effect on centriole re-duplication but is not involved in regulating the initial centriole assembly process. Therefore Tiam1 is crucial for limiting centriole duplication to once per cell cycle.

Figure 26. See figure legend p113.
4.1.4 The ability of Tiam1 to prevent centriole re-duplication is not dependent on Rac activation

This data suggests that Tiam1 has a regulatory role in preventing centriole re-duplication. Notably Tiam1 is a GEF which has been shown to exert its biological functions via the activation of the small Rho GTPase, Rac (Michiels et al., 1995). Rac has been implicated in several biological processes including the regulation of cell-cell adhesions, cell shape and polarity, cell survival and cell cycle progression (Boissier and Huynh-Do, 2014). There are limited studies showing Tiam1 can function independently of its ability to activate Rac (Minard et al., 2006; Otsuki et al., 2003), whereas Tiam1-mediated Rac signalling has been implicated in several biological processes (Boissier and Huynh-Do, 2014).

To determine whether Rac activation is necessary for the rescue of centriole amplification seen in Section 4.1.3, both Tiam1 WT-HA and Tiam1 GEF*-HA (a mutant of Tiam1 which is unable to activate Rac (Tolias et al., 2005)), were inducibly over-expressed in U2OS cells. Inducible over-expression was achieved using the two step pRetroXT TetON system (Clontech) (Figure 27).
Firstly, U2OS cells were retrovirally transduced with the pRetroX-Tet-On plasmid and stable expression was maintained by culturing cells in selection (G418). These cells were then retrovirally transduced with pRetroX-Tight-Pur plasmid containing either Tiam1 WT-HA or Tiam1 GEF*-HA. Retention of each construct was maintained by culturing in selection (G418; puromycin). The expression of each construct is controlled by a tetracycline responsive promoter (pTight). Upon the addition of DOX, the expression of rtTA from the pRetroX-Tet-On is stimulated – inducing expression of Tiam1 WT-HA and Tiam1 GEF*-HA. As each construct is HA-tagged, exogenous expression of Tiam1 can be detected by IF using an antibody against the HA protein (Figure 28).

Figure 27. Schematic of the DOX inducible expression system. The reverse tetracycline-controlled transactivator protein (rtTA) is constitutively expressed and unable to bind the pTight promoter in the absence of DOX. rtTA in combination with DOX binds pTight and drives expression of your gene of interest (GOI).
U2OS cells over-expressing Tiam1 WT-HA and Tiam1 GEF*-HA following the addition of DOX were treated for 4 days with hydroxyurea to induce centriole amplification. The exogenous expression of each construct was confirmed by western blot (Figure 29A). Hydroxyurea treatment results in a G1/S-phase arrest. As exogenous Tiam1 is HA tagged, HA positive cells were selected for the analysis (Figure 29B) (HA images correspond to images shown in Figure 29C). The centrioles were visualised by IF using centrin as a marker of the centrioles and pericentrin as a marker of the PCM (Figure 29C). Centriole number was quantified from IF images in control (-DOX) and cells over-expressing either Tiam1 WT-HA or Tiam1 GEF*-HA. Cells containing ≥5 centrioles were classified as aberrant. Notably, the over-expression of Tiam1 GEF* significantly decreased the number of cells with aberrant centriole number following hydroxyurea treatment (p=0.0019; 50 cells were quantified per experimental replicate; N=3) (Figure 29D; 29C). Significantly, the level of rescue is similar to that exerted by Tiam1 WT-HA (50 cells were quantified per experimental replicate; N=2) (Figure 29D; 29C). This data suggests that Tiam1 plays a role in preventing centriole re-duplication and this role is independent of its ability to activate Rac.
Figure 28. Inducible expression of Tiam1 WT and Tiam1 GEF*. The expression of Tiam1 WT-HA and Tiam1 GEF*-HA was induced by the addition of DOX. The expression of each construct was detected by IF using an HA antibody. Scale bar=10µm.

Figure 29. See figure legend p118.
Figure 29. See figure legend p118.
Figure 29. Over-expression of Tiam1 GEF* partially rescues centriole amplification caused as a result of hydroxyurea treatment. A) U2OS tumour cells were treated with 4mM of hydroxyurea for 4 days and exogenous expression of Tiam1 GEF*-HA and Tiam1 WT-HA was induced by the addition of DOX. Exogenous protein levels were detected by western blot using β Actin as a loading control. B) Exogenous protein levels were detected by IF using an HA antibody to visualise the tagged constructs. Images are a direct representation of images shown in C. Scale bar=10µm. C) Centrioles were visualised by IF using centrin as a marker of the centrioles and pericentrin as a marker of the PCM. Images are annotated with prediction of cell cycle phase based on centriole:centrosome ratio. Zoomed images are representative of boxed regions; scale bar=10µm. D) Centriole number was quantified in U2OS cells over-expressing Tiam1 WT-HA and Tiam1 GEF*-HA following hydroxyurea treatment. Cells containing ≥5 centrin signals were classed as aberrant. 50 cells were analysed per experimental replicate. P<0.01(**); bars depict standard error; Tiam1 GEF*-HA N=3; Tiam1 WT-HA N=2.
This experiment was replicated in the absence of hydroxyurea. Tiam1 WT-HA and Tiam1 GEF*-HA were exogenously expressed for 4 days by the addition of DOX. Exogenous protein levels were detected by western blot (Figure 29A). As exogenous Tiam1 is HA tagged, cells were selected for the analysis by staining for HA (Figure 30A) (HA images correspond to images shown in Figure 30B). Centriole number was assessed by IF using centrin as a marker of the centrioles and pericentrin as a marker of the centrosome (Figure 30B). Centriole number was quantified in control (-DOX) and Tiam1 over-expressing cells with cells containing ≥5 centrioles classed as aberrant. Over-expression of Tiam1 GEF*-HA in the absence of hydroxyurea has no significant effect on normal centriole assembly. This was replicated with Tiam1 WT-HA and supports previous data obtained in Section 4.1.3 (Tiam1 GEF*: p=0.4626; 50 cells analysed per experimental replicate; N=3, Tiam1 WT: 50 cells analysed per experimental replicate; N=2) (Figure 30C; 30B). Taken together this data suggests that Tiam1 and the downstream activation of Rac is not required for the initial centriole assembly process. However Tiam1 is important for preventing promiscuous centriole duplication events and this phenotype is potentially independent of its ability to activate Rac.

Figure 30. See figure legend p121.
Figure 30. See figure legend p121.
Figure 30. Over-expression of Tiam1 WT and Tiam1 GEF* has no effect on centriole duplication in the absence of hydroxyurea treatment. A) Tiam1 WT-HA and Tiam1 GEF*-HA were over-expressed by the addition of DOX for 4 days and protein levels were detected by IF using an HA antibody to visualise the tagged constructs. Images are a direct representation of images shown in B. Scale bar=10µm. B) Centriole number was visualised by IF using centrin as a marker of the centrioles and pericentrin as a marker of the PCM. Images are annotated with prediction of cell cycle phase based on centriole:centrosome ratio. Zoomed images are representative of boxed regions; scale bar=10µm. C) Centriole number was quantified in cells over-expressing Tiam1 WT-HA and Tiam1 GEF*-HA in the absence of hydroxyurea treatment. Cells containing ≥5 centrin signals were classed as aberrant. 50 cells were analysed per experimental replicate. NS=not significant; bars depict standard error; Tiam1 GEF*-HA N=3; Tiam1 WT-HA N=2.
4.2 Discussion and Future Plans

We reported in the previous chapter that loss of Tiam1 is sufficient to promote the assembly of supernumerary centriole structures in several human tumour cell lines. Tiam1 has previously been implicated in the centriole cycle, specifically regulating centrosome separation during mitosis (Whalley et al., 2015; Woodcock et al., 2010). However, Tiam1 has never been implicated in the regulation of centriole number. We have identified a novel role of Tiam1 in the centriole cycle – Tiam1 regulates centriole duplication. However the mechanism by which Tiam1 restrains centriole duplication is unknown. Centriole amplification can arise via two distinct mechanisms; indirectly via defective mitosis or directly by de-regulation of the centriole cycle. Loss of Tiam1 in human tumour cell lines must disrupt one of these processes to permit the assembly of aberrant centriole structures.

4.2.1 Aberrant centriole structures in Tiam1 depleted cells are not a bi-product of defective mitosis

As discussed, Tiam1 has been implicated in the regulation of centrosome separation (Whalley et al., 2015; Woodcock et al., 2010). It was shown that knockdown of Tiam1 causes an increase in centrosome separation and this causes a mitotic delay (Woodcock et al., 2010). Therefore, loss of Tiam1 in human cancer cell lines may be sufficient to promote centriole amplification as a result of defective mitosis. However loss of Tiam1 does not prevent mitotic exit in U2OS tumour cells. Knockdown of Tiam1 by siRNA for 3 days is sufficient to promote the assembly of supernumerary centrioles. However loss of Tiam1 for this duration did not have any effect on cell cycle progression and there was no correlation with an increased frequency of bi/poly-nucleate cells – providing evidence that cytokinesis is completed successfully in Tiam1 knockdown cells. This suggests that Tiam1 is not responsible for mitotic exit in U2OS tumour cells. This was expected as Woodcock et al., 2010 observed a prolonged mitosis following depletion of Tiam1 – not mitotic slippage. Therefore supernumerary centriole structures observed in Tiam1 knockdown cells are not the result of cytokinesis failure and defective mitosis.
4.2.2 Tiam1 prevents centriole re-duplication

As Tiam1 has no effect on mitotic exit, loss of Tiam1 must disrupt the regulation of the centriole cycle – permitting the assembly of supernumerary centrioles. Over-expression of Tiam1 was sufficient to significantly rescue the centriole amplification phenotype caused as a result of hydroxyurea treatment. Hydroxyurea causes centriole amplification by permitting the ectopic expression of CDK2 – a key regulator of centriole biogenesis (Matsumoto et al., 1999; Meraldi et al., 1999; Okuda et al., 2000). This uncouples the centriole cycle from the cell cycle, allowing multiple rounds of centriole duplication to occur within the same cell – i.e. the mother centriole produces a single daughter, they disengage, each disengaged centriole produces a single daughter and so on (Balczon et al., 1995; Tsang et al., 2009). As Tiam1 over-expression had no effect on normal centriole biogenesis, Tiam1 must specifically prevent promiscuous duplication events. This phenotype has also been shown for Cep76 – this protein specifically prevents centriole re-duplication events yet has no effect on normal centriole biogenesis (Tsang et al., 2009). This suggests that there is a group of proteins that are specifically involved in preventing promiscuous centriole duplication events when the centriole cycle becomes deregulated.

We have been examining the effect of Tiam1 depletion in human tumour cell lines – with particular focus on U2OS. Although there was no notable effect on cell cycle progression following the knockdown of Tiam1, this is not to say there is not an underlying defect in the U2OS cell cycle – particularly as it is a transformed cell line. Therefore U2OS may have an underlying defect in the cell cycle – independent of Tiam1 expression – that alters the expression pattern of key proteins involved in centriole biogenesis. Therefore the removal of Tiam1 by siRNA removes a key restraint, licensing promiscuous duplication events. Therefore it would be extremely interesting to determine whether loss of Tiam1 is sufficient to promote supernumerary centrioles in a non-transformed cell line. Loss of Tiam1 may not be sufficient to promote the assembly of aberrant centrioles in normal, untransformed cells.

Taken together this data suggests that loss of Tiam1 permits promiscuous duplication events, therefore Tiam1 is involved in limiting centriole duplication to once per cell cycle. To further support this hypothesis the effect of Tiam1 over-expression should be investigated in cells that produce an excessive number of daughter procentrioles – resulting in centriole amplification.
Centriole number is regulated by two mechanisms; centriole duplication is initiated once per cell cycle and centriole biogenesis is semi-conservative – a mother centriole is permitted to produce a single daughter procentriole (Nigg and Stearns, 2011). These two pathways are believed to be mechanistically distinct. Centriole copy number is regulated by PLK4 and over-expression of this protein is sufficient to produce an excessive number of procentrioles (Kleylein-Sohn et al., 2007). Interestingly over-expression of Cep76 – a negative regulator of centriole re-duplication – is insufficient to prevent centriole amplification caused by PLK4 over-expression (Tsang et al., 2009). This provides evidence that these two mechanisms of centriole amplification are distinct pathways. It would be interesting to explore this idea with Tiam1 – to determine whether over-expression of Tiam1 is sufficient to prevent centriole amplification caused as a result of PLK4 over-expression. Lastly as Tiam1 knockdown is sufficient to induce the formation of aberrant centriole structures in the following cell lines; U2OS, HCT116 and MCF7, the effect of over-expression of Tiam1 when treated with hydroxyurea should be investigated in both HCT116 and MCF7.

4.2.3 Tiam1 Prevents Centriole Re-Duplication Without the Downstream Activation of Rac

Tiam1 is a GEF that specifically activates Rac (Michiels et al., 1995). Tiam1-mediated Rac activation has been implicated in numerous biological processes with several studies linking alterations in this pathway to cancer (Boissier and Huynh-Do, 2014). From studies so far, the predominant role of Tiam1 is to activate Rac and this initiates a downstream signalling cascade that exerts a biological response. However there have been some studies that have shown a Rac independent role of Tiam1 (Minard et al., 2006; Otsuki et al., 2003). As we have identified Tiam1 as a novel regulator of the centriole cycle, it was clear we had to determine whether Rac was also implicated in this process. Most significantly, over-expression of Tiam1 GEF* (a mutant of Tiam1 that is unable to activate Rac (Tolias et al., 2005)) is still capable of rescuing the centriole amplification phenotype caused as a result of hydroxyurea treatment. This suggests that Tiam1 prevents centriole re-duplication independently of Rac activation. To this date there is no evidence to suggest that Rac activation by any GEF is required for centriole duplication.
There has been some evidence to suggest that centrosome amplification promotes activation of Rac and this correlates with an increased invasive phenotype (Godinho et al., 2014). However this has been linked to the increased MT nucleation capacity of cells with supernumerary centrosomes and not as a result of Rac promoting centriole amplification (Godinho et al., 2014). We have shown that over-expression of Tiam1 GEF* is sufficient to block centriole re-duplication, however this result alone is not sufficient to state that Tiam1-mediated Rac activation is not required for the regulation of centriole re-duplication. Levels of Rac activation should be quantified in cells treated with hydroxyurea to confirm that activation has been significantly inhibited. This experiment should also be repeated using constitutively active Rac. If Rac is not involved in regulating centriole re-duplication, over-expression of active Rac should be insufficient to prevent centriole amplification caused as a result of hydroxyurea treatment.

4.2.4 Conclusion

Taken together this data strongly suggests that Tiam1 is a negative regulator of the centriole cycle – specifically involved in limiting centriole duplication to once per cell cycle. Loss of Tiam1 licenses the assembly of multiple centriole structures, potentially in tumour cells that have an underlying defect within the cell cycle which makes them susceptible to centriole amplification. Preliminary data also suggests that Tiam1 exerts this restrictive role independently of the downstream activation of Rac. Combining the data presented in this chapter with the evidence presented in Chapter 3, it is clear that Tiam1 is a novel regulator of the centriole cycle.
Chapter 5. Tiam1 and MCM5 Co-operate to Prevent Centriole Re-duplication

5.1 Mechanism by which Tiam1 Prevents Centriole Re-duplication

5.1.1 Introduction

The data presented in Chapter 4 strongly suggests that Tiam1 has a regulatory role in the centriole cycle – more specifically Tiam1 prevents promiscuous duplication events. Several proteins have been identified as negative regulators of the centriole cycle (Brownlee and Rogers, 2013; Balestra et al., 2013). However the restrictive nature of these proteins remains unclear. Some have been shown to down-regulate the expression of key proteins required for centriole biogenesis – therefore their removal promotes the ectopic expression of proteins that positively enhance biogenesis (Puklowski et al., 2011). It is also thought that restrictive proteins may maintain the integrity of the S-M linker – presenting a centriole intrinsic block to duplication (Wong and Stearns, 2003). Therefore although it is widely accepted that centriole number is maintained by two broad mechanisms; ensuring duplication occurs once per cell cycle and duplication is semi-conservative, there is still much to understand with regards to regulating centriole number.

Tiam1 is a Rac specific GEF (Michiels et al., 1995), however we have shown that Tiam1 regulates centriole re-duplication without the downstream activation of Rac. Tiam1 is a large scaffolding protein that has been shown to interact with several proteins to mediate a biological response – some examples of Tiam1 interactors include syntrophin, Par3, Spinophilin and HUWE1 (Chen and Macara, 2005; Mack et al., 2012; Vaughan et al., 2015). Therefore Tiam1 may co-operate with additional protein(s) at the centrosome to regulate centriole number. Identification of centrosomal Tiam1 interactors may begin to elucidate the mechanism by which Tiam1 prevents centriole re-duplication.

5.1.2 Tiam1 Interacts with MCM5

As Tiam1 is a large scaffolding protein with multiple interaction domains (Figure 11), Tiam1 may co-operate with additional regulators of the centriole cycle to prevent centriole re-duplication. Previous mass spectrometry data suggested that Tiam1 interacts with the Mini-Chromosome Maintenance Factor 5 (MCM5) (Mack, 2010).
MCM5 belongs to the MCM family of chromatin binding proteins which are classically known for their involvement in DNA replication. Multiple members of the MCM family (MCM2-7) interact to form the MCM complex, a large hexamer that envelopes the DNA double helix (Lei, 2005). This complex sits at origins of replication and unwinds DNA allowing the initiation of DNA replication (Lei, 2005). The timed dissociation of the MCM complex from DNA is regarded as a key step in insuring DNA replication is licensed once per cell cycle (Lei, 2005). The MCM5 protein is small in comparison to Tiam1 and has one main functional domain – the MCM box. This domain is important for ATP binding and is conserved throughout the MCM protein family members (Figure 31) (Forsburg, 2004). Significantly MCM5 has been shown to act independently of the MCM complex, with a role in regulating the centriole cycle. MCM5 has been shown to act negatively on centriole duplication, specifically preventing centrosome re-duplication (Ferguson and Maller, 2008). This mirrors the phenotype that we have suggested for Tiam1. Therefore MCM5 was a prime candidate for further study.

The interaction between Tiam1 and MCM5 was validated by IP from lysates collected from asynchronous U2OS tumour cells (N=2) (Figure 32A). This suggests that MCM5 is a true Tiam1 interactor. In the previous chapter we have shown that Tiam1 over-expression is sufficient to partially rescue centriole amplification caused as a result of hydroxyurea arrest, suggesting that Tiam1 is specifically involved in preventing centriole re-duplication in S-phase. Additionally MCM5 has been identified as a negative regulator of aberrant centrosome re-duplication (Ferguson and Maller, 2008). Therefore the interaction between Tiam1 and MCM5 may be more prominent when providing the opportunity of promiscuous centriole duplication events. Therefore the interaction between Tiam1 and MCM5 was examined following partial S-phase arrest by a double thymidine block. Treatment with thymidine halts the dNTP synthesis pathway, resulting in a G1/S-phase arrest (Harper, 2005).

Figure 31. Schematic of the MCM5 protein. The main functional domain in MCM5 is the MCM box. This region is responsible for ATP binding.
Figure 32. See figure legend p129.
U2OS cells were treated with a double thymidine block and partial cell cycle arrest was confirmed by flow cytometry ($\geq 1 \times 10^4$ cells were analysed per experimental; N=2) (Figure 32B). Lysates were collected from thymidine treated U2OS cells and the interaction between Tiam1 and MCM5 was examined by IP (N=2) (Figure 32C). The interaction between Tiam1 and MCM5 intensified following partial S-phase arrest in U2OS cells. This further supports the hypothesis that Tiam1 is important for preventing promiscuous centriole duplication events. We have shown that Tiam1 interacts with MCM5 in the U2OS human tumour cell line. However, as shown in Section 3.1.2 Tiam1 knockdown is sufficient to promote the assembly of aberrant centriole structures in another two human tumour cell lines; HCT116 and MCF7. Therefore the interaction between Tiam1 and MCM5 was investigated in HCT116. The interaction between Tiam1 and MCM5 was confirmed by IP from asynchronous lysates collected from HCT116 (N=2) (Figure 32D). Therefore the interaction between Tiam1 and MCM5 is not restricted to one cell line. As Tiam1 and MCM5 both prevent centriole re-duplication this interaction may be important for the regulation of centriole number.
5.1.3 Determining the MCM5 binding region in Tiam1

We have shown that MCM5 is a novel Tiam1 interactor and we believe this interaction could be important for preventing centriole re-duplication. To confirm our hypothesis the domains that mediate the interaction between Tiam1 and MCM5 must be identified. Following the identification of an MCM5 binding region, a Tiam1 mutant can be generated that is unable to bind MCM5. The behaviour of this mutant will determine whether the Tiam1-MCM5 interaction is important for preventing centriole re-duplication.

In the laboratory we have various Tiam1 truncation mutants. Each mutant is in a pcDNA3.1 backbone and is constitutively expressed by a pCMV promoter. The following constructs were transiently transfected in to HEK293T cells; Tiam1 WT-HA, C1199-HA, C580-HA, C196-HA, KETDI 44-HA, M4-HA and PHCCEx-HA (Figure 33).

Figure 33. Schematic of Tiam1 truncation mutants. Each truncation mutant is HA tagged at the C terminus. PESTs: PEST sequence, PHnCCEx: N-terminal plekstrin homology, coiled coil and extended domain, RBD: Ras binding domain, PDZ; PSD-95, Dlg1, ZO-1/2 domain, DH: Dbl homology domain, PHc: C-terminal plekstrin homology domain, KETDI: KETDI amino acid sequence.
HEK293T cells were selected for this experiment because they drive high expression of exogenous protein when using the pcDNA3.1 backbone. Each construct was immunoprecipitated by HA and MCM5 interactions were determined by western blot (N=5) (Figure 34). MCM5 was capable of interacting with multiple Tiam1 domains – including N terminal and C terminal regions. Given this surprising result, it was hypothesised that the interaction between Tiam1 and MCM5 was indirect. Tiam1 and MCM5 may associate in a protein complex and this might explain why an interaction can be detected with multiple Tiam1 domains. Therefore the interaction between Tiam1 and MCM5 was assessed in vitro.

Figure 34. Determining the interaction between MCM5 and various Tiam1 truncation mutants. Tiam1 truncation mutants were transiently transfected in to HEK293T cells and each mutant was immunoprecipitated by HA. A positive MCM5 interaction was detected by western blot. Immunoblots are representative of N=4.
5.1.4 The interaction between Tiam1 and MCM5 is direct

We have shown that Tiam1 and MCM5 interact. However this interaction was validated by IP from lysates collected from cell lines – this method does not confirm whether Tiam1 and MCM5 directly interact. Using whole cell lysates leaves the possibility that Tiam1 and MCM5 interact indirectly via an intermediate protein. Therefore to determine whether Tiam1 and MCM5 are capable of interacting directly, an *in vitro* GST pulldown assay was conducted. The HECT domain of HUWE1 is a known direct interactor of Tiam1 and was therefore used as a positive control for the *in vitro* assay (Vaughan et al., 2015). MCM5-GST protein was purified from bacteria and purified HECT-GST and Tiam1-His were kindly donated by Lynsey Vaughan and Chong Teik Tan – previous members of the laboratory. An interaction between purified MCM5-GST and Tiam1-His was validated by IP. Immunoprecipitated MCM5-GST is able to coimmunoprecipitate Tiam1-His significantly more than a GST only control (N=2) (*Figure 35*). Therefore Tiam1 and MCM5 can interact *in vitro*. This strongly suggests that MCM5 is a direct interactor of Tiam1.

*Figure 35. Tiam1 interacts with MCM5 *in vitro*. Purified Tiam1-His was incubated with purified MCM5-GST in an *in vitro* GST pull-down assay. Purified HECT-GST was used as a positive control. Positive interactions were detected by western blot. Boxes indicate correct sized bands corresponding to each GST tagged protein.*
5.1.5 The N terminal region of Tiam1 is responsible for its interaction with MCM5 \textit{in vitro}

Tiam1 and MCM5 can directly interact \textit{in vitro} however we were unable to identify a truncation mutant of Tiam1 that is unable to bind MCM5 (Section 5.1.3). We hypothesised that this may be because there are multiple MCM5 binding sites within the Tiam1 protein. Therefore we set up an external collaboration to fine map the MCM5 binding region(s) within Tiam1.

Professor George Ballie’s laboratory (University of Glasgow) generated a Tiam1 peptide array. A peptide array is an \textit{in vitro} technique, a collection of peptides are generated that correspond to a protein of interest. These peptides are hybridised to a nitrocellulose membrane and probed with purified protein. The purified protein will bind to peptides that are capable of mediating an interaction \textit{in vitro} and positive interactions are detected by immunoblotting. \textbf{Figure 36} is a schematic of the Tiam1 peptide array workflow.
Figure 36. Schematic of the peptide array workflow. Peptides are generated that walk along the full length of Tiam1. These peptides are hybridized to an array which is then probed with purified MCM5 protein. Positive interactions are detected by immunoblotting with MCM5 antibody.
Peptides corresponding to the Tiam1 WT sequence (human) were hybridized in duplicate to a nitrocellulose membrane. A list of each peptide sequence and the arrangement of the array can be found in Appendix Table 1; Figure 1. The array was probed with purified MCM5-GST protein and positive interactions were detected with MCM5 antibody (Figure 37A). A positive interaction was identified with peptide 48. This was replicated in duplicate and no positive interactions were identified in the negative controls (GST only; antibody only) (Figure 37B). Peptide 48 corresponds to a 25 amino acid region in the N terminus of Tiam1 (Figure 38). Our results suggest that this region is sufficient to mediate an interaction between Tiam1 and MCM5 in vitro.

Figure 37. See figure legend p136.
5.1.6 Loss of the putative MCM5 binding region is not sufficient to completely abolish MCM5 binding

A putative MCM5 binding site was identified in the N terminus of Tiam1 by peptide array. To confirm that this region is required to mediate an interaction with MCM5 in cells, a Tiam1 mutant was generated that lacks the putative binding site. Primers were designed that flank the putative MCM5 binding region and using site directed mutagenesis this region was deleted from Tiam1 WT-HA (Figure 39). The site directed mutagenesis protocol was carried out by Gavin White, the scientific officer of our laboratory. Tiam1 WT-HA was used as a template for the mutagenesis protocol because it is in the DOX inducible pRetroX-Tight-Pur system (Clontech) (discussed in Section 4.1.4). Therefore the putative MCM5 binding mutant – Tiam1 Δ25 – is HA tagged and can be over-expressed by the addition of DOX. A U2OS cell line that inducibly expresses Tiam1 Δ25-HA was made as outlined in Section 4.1.4.

Figure 37. Peptide mapping of the putative MCM5 binding region. A) The array was probed with purified MCM5-GST protein and positive interactions were determined by immunoblotting with MCM5 antibody. Purified GST protein was used as a negative control. Red box corresponds to a positive interaction with MCM5. B) Zoom of region highlighted in A.

Figure 38. The putative MCM5 binding region is located in the N terminal region of Tiam1. Diagrammatic representation of peptide 48 identified in Figure 37. Peptide 48 corresponds to a 25 amino acid region found within the N terminal region of Tiam1.
The interaction between Tiam1 Δ25-HA and endogenous MCM5 was assessed by IP. MCM5 was immunoprecipitated from lysates collected from U2OS cells over-expressing Tiam1 Δ25-HA. U2OS cells expressing Tiam1 WT-HA were used as a positive control. Tiam1 Δ25-HA can still interact with MCM5 (Figure 40A). As we have shown that the interaction between Tiam1 and MCM5 intensifies following thymidine treatment (Section 5.1.2) this experiment was repeated in the presence of thymidine. However Tiam1 Δ25-HA can still interact with MCM5 (Figure 40B). This data suggests that the 25 amino acid region identified by the peptide array is not the predominant MCM5 binding site in Tiam1.

Figure 39. Generation of a putative MCM5 binding mutant. Primers were designed that flank the putative MCM5 binding site. Using Site Directed Mutagenesis this region was selectively deleted by PCR.
Figure 40. The putative MCM5 binding mutant can still interact with MCM5. A) Tiam1 WT-HA and Tiam1 Δ25-HA were over-expressed for 3 days by the addition of DOX. Endogenous MCM5 was immunoprecipitated from collected lysate and a potential interaction with each construct was detected by western blot. Immunoblots are representative of N=5. B) Tiam1 WT-HA and Tiam1 Δ25-HA were over-expressed for 3 days by the addition of DOX and cells were treated with a double thymidine block (18 hours 2mM thymidine; 8 hours release; 17 hours 2mM thymidine). Endogenous MCM5 was immunoprecipitated from collected lysates and a potential interaction with each construct was detected by western blot. Immunoblots are representative of N=5.
5.2 Investigating the biological significance of the Tiam1 and MCM5 interaction

5.2.1 Introduction

While we were trying to determine the MCM5 binding site within Tiam1, experiments were run in parallel to determine the biological significance of the Tiam1-MCM5 interaction. The data presented in Chapter 4 strongly suggests that Tiam1 regulates centriole number by preventing centriole re-duplication. We have shown that Tiam1 interacts directly with MCM5. MCM5 also regulates centriole number – specifically preventing centrosome re-duplication (Ferguson and Maller, 2008). Given this, Tiam1 and MCM5 may co-operate to regulate centriole re-duplication and the direct interaction between these proteins may be necessary to exert this phenotypic effect.

5.2.2 Tiam1 and MCM5 localise to the centrosome

MCM5 has been shown to localise to the centrosome and this localisation is required for preventing centrosome re-duplication (Ferguson and Maller, 2008). Therefore to elucidate the mechanism by which Tiam1 and MCM5 co-operate to regulate centriole number, their co-localisation was initially examined in U2OS tumour cells. As it is possible to generally predict cell cycle phase based on centriole number, the localisation of Tiam1 and MCM5 could be examined in each cell cycle phase. Tiam1 and MCM5 were visualised at the centrioles during G1 phase. Tiam1 appears as a haze potentially associated with PCM while MCM5 appears to associate with one centriole within the pair (Figure 41). Tiam1 and MCM5 were also located at the centrioles during S/G2 phase. Again Tiam1 appears to behave like a PCM marker with two distinct regions of Tiam1 localised to each centriole pair. There are two MCM5 signals and each appears to associate with a single centriole within a duplicated pair (Figure 42). Tiam1 and MCM5 were also present at the centrioles during mitosis. Tiam1 is associated with the spindle poles and resembles a PCM marker. The MCM5 signal has re-distributed with discrete signals associated with each centriole within a pair (Figure 43). Taken together this data suggests that Tiam1 may be preferentially associated with the PCM while MCM5 localises more specifically to the centrioles. This data also shows that Tiam1 and MCM5 are localised at the centrioles throughout interphase and mitosis. This further suggests that Tiam1 and MCM5 co-operate at the centrioles to restrain centriole re-duplication.
Figure 41. Tiam1 and MCM5 localise at the centrioles in G1 Phase cells. The localisation of Tiam1 and MCM5 was visualised in U2OS tumour cells by IF. Centrin was used as a marker of the centrioles. Images are annotated with prediction of cell cycle phase based on centriole number and schematic of predicted Tiam1:MCM5:centrin relationship. Zoomed images are representative of boxed regions; scale bar=10µm.
Figure 42. Tiam1 and MCM5 localise at the centrioles in S/G2 Phase cells. The localisation of Tiam1 and MCM5 was visualised in U2OS tumour cells by IF. Centrin was used as a marker of the centrioles. Images are annotated with prediction of cell cycle phase based on centriole number and schematic of predicted Tiam1:MCM5:centrin relationship. Zoomed images are representative of boxed regions; scale bar=10µm.
Figure 43. Tiam1 and MCM5 localise at the centrioles in Mitotic cells. The localisation of Tiam1 and MCM5 was visualised in U2OS tumour cells by IF. Centrin was used as a marker of the centrioles. Images are annotated with prediction of cell cycle phase based on centriole number and schematic of predicted Tiam1:MCM5:centrin relationship. Zoomed images are representative of boxed regions; scale bar=10µm.
5.2.3 Determining the effect of Tiam1 knockdown on MCM5 localisation

As previous data has shown, loss of Tiam1 is sufficient to promote the assembly of aberrant centriole structures in multiple human tumour cell lines. Given that Tiam1 and MCM5 directly interact and both proteins localise to the centrioles throughout the cell cycle, loss of Tiam1 may disrupt the localisation of MCM5 to the centrioles.

5.2.3a siRNA knockdown of Tiam1 and the effect on MCM5 localisation

To determine whether Tiam1 knockdown has an effect on the localisation of MCM5, we planned to deplete Tiam1 by siRNA and analyse MCM5 localisation at the centrioles. Tiam1 was knocked down for 3 days by siRNA and a significant reduction in protein levels was confirmed by western blot (Figure 44A). When examining Tiam1 levels at the centrioles by IF, following treatment with siRNA there is a reduction in Tiam1 signal when compared to control (NT siRNA), however residual Tiam1 protein is still detectable at the centrioles (Figure 44B).

Figure 44. See figure legend p144.
This has been a recurring problem in the laboratory. Treatment with siRNA significantly reduces protein levels detected by western blot however protein is still detectable at the centrioles by IF. This may be due to the sensitivity of IF, siRNAs are not 100% penetrant and even a small pool of residual protein may be sufficient to generate a detectable signal. It is also possible that Tiam1 is stably incorporated within the centrosome, making it challenging to completely eradicate the protein from this organelle. Given the underlying challenges of this experiment we decided to use newly developed CRISPR technology to completely deplete endogenous levels of Tiam1.

5.2.3b CRISPR genome editing of Tiam1

It is now possible to create total gene knockouts in cell lines using CRISPR technology. CRISPR is a recently developed technique that utilises the immune response of *Streptococcus* (Sander and Joung, 2014). Cas9 is a nuclease that can be targeted to a specific genomic region by the design of a unique guide RNA (gRNA). Cas9 selectively cleaves this target DNA, resulting in DNA double stranded breaks. This DNA damage will be repaired by non-homologous end joining during recombination, resulting in frameshift deletions and/or insertions. This can result in a non-functional gene product.

Our aim was to produce a cell line that is a complete Tiam1 knockout. However, long term culture of Tiam1 knockout cells might promote the up-regulation of pathways that compensate for the lack of Tiam1 protein. Therefore it was decided to perform CRISPR in U2OS cells that exogenously express Tiam1 WT-HA following the addition of DOX – Tiam1 knockout cells would be cultured long term in the presence of DOX.
To remove endogenous Tiam1, CRISPR technology was used to interrupt the Tiam1 gene locus. A gRNA that targets Cas9 to the Tiam1 gene locus was designed by Zoi Diamantopoulou, a post doc in the laboratory (Figure 45A). U2OS cells transfected with Tiam1 gRNA and Cas9 were single cell sorted by GFP (this selects cells that are positive for the CRISPR construct). Individual clones were screened for Tiam1 expression following the removal of DOX for 3 days. Based on endogenous Tiam1 expression levels, clone 13 was selected for further analysis (Figure 45B). To determine whether this clone was a complete genomic knockout of Tiam1, the genomic region targeted by the Tiam1 gRNA was sequenced. Sequencing analysis revealed that one allele had a frameshift deletion and the other allele had a deletion that did not result in a frameshift mutation (Figure 45C). Therefore it is likely that clone 13 is heterozygous at the Tiam1 gene locus. However as the expression of endogenous Tiam1 was extremely low (Figure 45B), this clone was taken forward in further experiments. From this point clone 13 will be referred to as U2OS Tiam1\(^{+/−}\).

Data presented in Section 5.2.3b was kindly provided by Zoi Diamantopoulou, a post doc in the laboratory and Gavin White (scientific officer).

![Diagram of CRISPR process](image)

**Figure 45.** See figure legend p146.
Figure 45. Generation of Tiam1 knockout clones by CRISPR. A) Schematic of the region targeted by Cas9 within the Tiam1 gene locus. The sgRNA target sequence requires a PAM sequence (5’-NGG-3’), this is required for caspase cleavage. B) Screening of CRISPR clones by western blot using β Actin as a loading control. Clone 13 was selected for further analysis. C) Sequencing results of Clone 13. Region sequenced corresponds to targeted region of the Tiam1 gene locus.

Data presented in this figure was kindly provided by Zoi Diamantopoulou, a post doc in the laboratory, and Gavin White (scientific officer).
5.2.3c MCM5 localisation in U2OS Tiam1+/− cells

U2OS Tiam1+/− cells generated by CRISPR technology express low levels of endogenous Tiam1. These cells also inducibly express Tiam1 WT-HA following the addition of DOX. To determine whether Tiam1 plays a role in recruiting MCM5 to the centrioles, MCM5 localisation was analysed in control (-DOX) and Tiam1 over-expressing cells.

DOX was removed from U2OS Tiam1+/− cells for a minimum of 7 days and protein levels were detected by western blot (Figure 46A). The localisation of MCM5 and Tiam1 was visualised in control (-DOX) and Tiam1 WT-HA over-expressing cells (+DOX) by IF (Figure 46B). The fluorescence intensity of Tiam1 and MCM5 at the centrioles was quantified from IF images using Image J software. A region of interest (ROI) was drawn around the centrioles and a fluorescence intensity value was determined from this region. The ROI – shape and area – was kept consistent throughout the experiment (Figure 46C). Using this method the intensity of MCM5 signal at the centrioles was quantified in control (-DOX) and Tiam1 WT-HA over-expressing cells. Following over-expression of Tiam1 there is a modest increase in the fluorescence intensity of MCM5 at the centrioles (p=0.1105; 50 pairs of centrioles quantified per experimental replicate; N=5) (Figure 46D). A similar increase was observed with Tiam1 (p=0.1541; 50 pairs of centrioles quantified per experimental replicate; N=5) (Figure 46E). This suggests that over-expression of Tiam1 can increase the levels of MCM5 protein at the centrioles. Notably Tiam1 over-expression had no effect on the global levels of MCM5 (Figure 46F). Therefore the modest increase in MCM5 intensity must be due to a change in localisation of the protein. Taken together this preliminary data suggests that Tiam1 can influence the localisation of MCM5 at the centrioles. However the increase in MCM5 localisation was not significant, therefore it is unclear whether Tiam1 is responsible for the recruitment of MCM5 at the centrioles.
Figure 46. See figure legend p150.
Figure 46. See figure legend p150.
Figure 46. Over-expression of Tiam1 correlates with a modest increase of MCM5 signal at the centrioles. A) Tiam1 WT-HA was over-expressed by the addition of DOX in a cell line that expresses endogenously low levels of Tiam1 (U2OS Tiam1<sup>+/−</sup>). Exogenous and endogenous Tiam1 levels were detected by western blot using β Actin as a loading control. B) The localisation of Tiam1 and MCM5 was visualised in control (-DOX) and Tiam1 over-expressing cells by IF. Centrin was used as a marker of the centrioles. Images are annotated with prediction of cell cycle phase. Zoomed images are representative of boxed regions; scale bar=10µm. C) Example of calculating fluorescence intensity value using Image J software. Zoom image is representative of boxed region; scale bar=10µm. D) The fluorescence intensity of MCM5 at the centrioles was quantified in control (-DOX) and Tiam1 over-expressing cells using Image J software as outlined in C. Raw data values obtained from Tiam1 over-expressing cells were normalised to the average fluorescence intensity measured from control cells (-DOX). Scatter plots contain values obtained from 5 independent experiments with 50 centrioles analysed per experimental replicate. E) The fluorescence intensity of Tiam1 at the centrioles was quantified in control (-DOX) and Tiam1 over-expressing cells using Image J software as outlined in C and D. Scatter plots contain values obtained from 5 independent experiments with 50 centrioles analysed per experimental replicate. F) The expression of MCM5 in control (-DOX) and Tiam1 over-expressing cells was determined by western blot using β Actin as a loading control.
5.2.4 Determining the effect of MCM5 knockdown on Tiam1 localisation

Preliminary data suggests that Tiam1 can influence the localisation of MCM5 at the centrioles. However MCM5 may affect the localisation of Tiam1 at the centrioles. Therefore we decided to assess the effect of MCM5 knockdown on Tiam1 localisation. Three siRNAs were designed against the MCM5 mRNA (Figure 47A). These siRNAs were used to knockdown MCM5 for 3 days in U2OS cells and protein levels were detected by western blot. However there was no reduction in MCM5 protein when compared to control (NT siRNA) (Figure 47B). This may be explained by the necessity of the MCM5 protein for viability. MCM5 is a component of the MCM complex which is essential for licensing DNA replication (Lei, 2005). A significant reduction in the MCM5 protein has been shown to cause cell cycle arrest (Ryu et al., 2005). Therefore investigating the functional significance of the Tiam1-MCM5 interaction via the knockdown of MCM5 is likely to be difficult.

Figure 47. MCM5 knockdown in U2OS tumour cells. A) Schematic of the MCM5 protein and target sites of MCM5 #1, #2 and #3 siRNA. B) MCM5 was knocked down for 3 days using MCM5 siRNA #1, #2 and #3. Protein levels were determined by western blot using β Actin as a loading control.
5.2.5 The putative MCM5 binding mutant cannot significantly block centriole re-duplication

The data presented in this chapter shows that Tiam1 and MCM5 interact however the biological significance of this interaction remains unclear. We hypothesise that this interaction is important for preventing centriole re-duplication. Therefore in parallel to experiments described in Section 5.1 we investigated whether the putative MCM5 binding mutant is capable of preventing centriole re-duplication in cells treated with hydroxyurea. Even though we have shown that this is not the predominant MCM5 binding site within Tiam1, this region is sufficient to support an interaction between Tiam1 and MCM5 in vitro. Therefore Tiam1 ∆25 may behave differently to Tiam1 WT.

U2OS cells which inducibly over-express Tiam1 ∆25-HA following the addition of DOX were treated with hydroxyurea for 4 days. Previous data has shown that over-expression of Tiam1 WT-HA is sufficient to partially rescue the centriole amplification phenotype caused as a result of hydroxyurea treatment (Section 4.1.3; 4.1.4). Therefore Tiam1 WT-HA was included in the experiment as a positive control. Exogenous protein levels were detected by western blot (Figure 48A). As each construct is HA tagged, cells were selected for the analysis based on the detection of HA expression by IF (Figure 48B) (HA images correspond to images shown in Figure 48C).

Figure 48. See figure legend p154.
Figure 48. See figure legend p154.
Centrioles were visualised by IF in hydroxyurea treated cells using centrin as a marker of the centrioles and pericentrin as a marker of the PCM (Figure 48C). Centriole number was quantified from IF images and cells containing ≥5 centrioles were classed as aberrant.
Over-expression of the putative MCM5 binding mutant (Tiam1 Δ25-HA) was unable to significantly reduce the number of cells with aberrant centrioles when compared to control (-DOX) (Tiam1 Δ25: p=0.1370; 50 cells examined per experimental replicate, N=4) (Figure 48D). In contrast, over-expression of Tiam1 WT-HA significantly reduces the number of cells with aberrant centriole structures when compared to control (-DOX) (Tiam1 WT: p=0.0085; 50 cells examined per experimental replicate; N=4). This is in support of previous data presented in Chapter 4. This data suggests that the putative MCM5 binding mutant behaves differently to Tiam1 WT.

This experiment was replicated in the absence of hydroxyurea. Tiam1 Δ25-HA and Tiam1 WT-HA were over-expressed for 4 days by the addition of DOX and exogenous protein levels were detected by western blot (Figure 48A). Cells were selected for the analysis based on levels of HA expression detected by IF (Figure 49A) (HA images correspond to images shown in Figure 49B). Centrioles were visualised by IF in cells over-expressing Tiam1 Δ25-HA and Tiam1 WT-HA using centrin as a marker of the centrioles and pericentrin as a marker of the PCM (Figure 49B). Centriole number was quantified from IF images and cells containing ≥5 centrioles were classed as aberrant (Figure 49C). Over-expression of Tiam1 Δ25-HA has no significant effect on centriole assembly (Tiam1 Δ25: p=0.6918; 50 cells per experimental replicate; N=3). This was also the case when over-expressing Tiam1 WT-HA, which is in support of previous data (see Chapter 4) (Tiam1 WT: p=0.1161; 50 cells per experimental replicate; N=3). This data suggests that the putative MCM5 binding mutant has no effect on normal centriole biogenesis, a result which has been replicated with Tiam1 WT.

![Figure 49. See figure legend p157.](image-url)
Figure 49. See figure legend p157.
Figure 49. Over-expression of Tiam1 Δ25 has no effect on centriole duplication in the absence of hydroxyurea treatment. A) Tiam1 Δ25-HA and Tiam1 WT-HA were over-expressed by the addition of DOX and protein levels were detected by IF using an HA antibody to visualise the tagged constructs. Images are a direct representative of images shown in B. Scale bar=10µm. B) Centrioles were visualised by IF using centrin as a marker of the centrioles and pericentrin as a marker of the PCM. Images are annotated with prediction of cell cycle phase based on centriole:centrosome ratio. Zoomed images are representative of boxed regions; scale bar=10µm. C) Centriole number was quantified in cells over-expressing Tiam1 Δ25-HA and Tiam1 WT-HA in the absence of hydroxyurea. Cells containing ≥5 centrin signals were classed as aberrant. 50 cells were analysed per experimental replicate. NS=not significant; bars depict standard error; N=3.
5.3 Discussion and Future Plans

5.3.1 Validating MCM5 as a *bona fide* interactor of Tiam1

Data presented in Chapter 4 strongly suggests that Tiam1 is involved in preventing centriole re-duplication – ensuring centriole duplication occurs once per cell cycle. Preliminary data also suggests that Tiam1 prevents centriole re-duplication by an unknown mechanism that is independent of Rac activation. As Tiam1 is a large scaffolding protein with multiple interaction domains, it is possible that Tiam1 interacts with other proteins that are centrosomally localised – identifying these interactions could elucidate the mechanism by which Tiam1 prevents centriole re-duplication.

We have identified MCM5 as a novel interactor of Tiam1 – in cell lines and *in vitro*. MCM5 is centrosomally localised and has been shown to prevent centrosome re-duplication (Ferguson and Maller, 2008). We were able to show that endogenous Tiam1 and endogenous MCM5 interact in U2OS and HCT116 cell lines – both of these cell lines show a significant increase in aberrant centriole number following Tiam1 knockdown. Therefore the interaction between Tiam1 and MCM5 may be important for regulating centriole number. In support of this we have shown that the Tiam1-MCM5 interaction is stronger in cells that are partially arrested at the G1-S phase boundary. This suggests that this interaction is important in conditions that promote promiscuous duplication events. Taken together this data strongly suggests that MCM5 is a *bona fide* interactor of Tiam1 and these proteins co-operate to prevent centriole re-duplication.

5.3.2 Centriole vs. centrosome

Ferguson and Maller (2008) identified MCM5 as a novel regulator of centrosome number while we have been investigating the effect of Tiam1 on centriole number. However the data presented by Ferguson and Maller does not contradict the potential involvement of MCM5 in regulating centriole number in partnership with Tiam1. The centrioles form the core of the centrosome – the centrioles recruit PCM and therefore dictate the duplication of the centrosome (Basto et al., 2006; Bobinnec et al., 1998). Over-expression of MCM5 was shown to reduce the number of γ tubulin signals (a recognised PCM marker (Moritz et al., 2000)) following treatment with hydroxyurea (Ferguson and Maller, 2008).
Hydroxyurea induces centriole amplification which in turn promotes centrosome amplification (Balczon et al., 1995; Matsumoto et al., 1999). Therefore although Ferguson and Maller (2008) documented MCM5 as a regulator of centrosome number - due to experimental design, this is analogous with a regulator of centriole number.

5.3.3 Defining the MCM5 binding region

It is clear that Tiam1 and MCM5 interact and that both proteins are implicated in the regulation of centriole duplication. However determining whether Tiam1 and MCM5 cooperate to prevent centriole re-duplication requires the identification of an MCM5 binding region within Tiam1. Characterisation of an MCM5 binding mutant will determine the biological significance of this interaction.

We could not identify a region of Tiam1 that was unable to bind MCM5. Initially IP experiments were carried out to try and define the region of Tiam1 responsible for MCM5 binding. However several Tiam1 truncation mutants were capable of interacting with MCM5 – covering both N terminal and C terminal regions. As this experiment was conducted using lysates from HEK293T cells it was hypothesised that the interaction between Tiam1 and MCM5 was indirect. It is possible that Tiam1 and MCM5 interact as part of a multi-protein complex – allowing MCM5 to interact with various Tiam1 truncation mutants. However Tiam1 and MCM5 interact in vitro and a putative MCM5 binding site was identified within the N terminal region of Tiam1 by peptide array. A putative MCM5 binding mutant was generated and we were able to show that this mutant is still capable of interacting with MCM5. This has now been independently verified by an additional two members of our laboratory. This data strongly suggests that the region identified by the peptide array is not the predominant MCM5 binding site within Tiam1. This could be explained by the following;

- Firstly a peptide array is an \textit{in vitro} assay. Although this region is sufficient to mediate an interaction \textit{in vitro} this may not be the predominant binding site in cells. Particularly as there was a significant amount of background on the peptide array, it is possible that additional binding sites are being masked in this assay. Multiple MCM5 binding sites would explain why several Tiam1 truncation mutants are capable of interacting with MCM5.
Therefore the peptide array should be optimised. It would be beneficial to carry out 3D modelling of the Tiam1 protein. The conformation of Tiam1 may provide insight into potential MCM5 binding region(s). A conformational change within the protein – potentially initiated at times of cell cycle stress – could bring an additional binding site in close proximity to the 25 amino acid region identified by the peptide array. Together these sites could promote binding to MCM5 (Figure 50). This could explain why removal of this single putative MCM5 binding site is insufficient to prevent an interaction with MCM5.

![Figure 50. Model 1 - Tiam1 contains multiple MCM5 binding sites. Optimal binding to MCM5 may require a conformational change within Tiam1 which brings two MCM5 binding sites (M1, M2) in close proximity. Removal of the putative MCM5 binding site (M1) may still facilitate binding.](image)

- Although Tiam1 and MCM5 are sufficient to bind in vitro, this does not fully replicate the biological situation in cells. Tiam1 and MCM5 may associate directly however they may be incorporated within a protein complex that is important for regulating centriole number. When Ferguson and Maller (2008) identified MCM5 as a regulator of centosome re-duplication they also identified Cyclin E as a direct interactor of MCM5. MCM5 binds directly to a centrosome localisation signal (CLS) within Cyclin E and binding to this region is important for the localisation of both proteins to the centrosome (Ferguson and Maller, 2008). Therefore Tiam1-MCM5 and MCM5-Cyclin E may co-operate in a complex that is important for preventing centriole re-duplication (Figure 51). This could be hindering the identification of MCM5 binding mutant in cells. Therefore it is important to determine whether Cyclin E can interact with Tiam1.
Taken together there are several avenues that can be investigated to finally identify the region(s) within Tiam1 that are important for mediating an interaction with MCM5.

![Diagram](image)

**Figure 51. Model 2 - Tiam1 interacts with Cyclin E.** Tiam1 might form a complex with MCM5 and Cyclin E. Deletion of the putative MCM5 binding site (M1) may prevent a direct interaction with MCM5 but MCM5 can still associate with Tiam1 via Cyclin E.

5.3.4 The MCM5 binding mutant behaves differently to Tiam1 WT

Functional assays were run in parallel to the biochemical assessment of the putative MCM5 binding region. It is clear from the biochemical data that the 25 amino acid region identified by the peptide array is not the predominant MCM5 binding site within Tiam1. However it was interesting to see that the Tiam1 Δ25 mutant behaved differently from Tiam1 WT in hydroxyurea treated cells. Over-expression of Tiam1 Δ25 could not block centriole re-duplication to the same extent as Tiam1 WT – suggesting that the Δ25 region plays a role in preventing promiscuous duplication events. Although Tiam1 Δ25 could not significant reduce the number of cells with aberrant centriole number, there was still a reduction when compared to control (15% reduction when compared to control). Therefore the Tiam1 Δ25 mutant is still causing a restrictive effect. This result may support the hypothesis that there are two MCM5 binding sites within Tiam1. Depletion of one may cause a reduction in MCM5 associated with Tiam1 and this may hinder the re-duplication block – explaining why there was not a significant effect when over-expressing Tiam1 Δ25 in hydroxyurea treated cells.
5.3.5 Determining whether Tiam1 and MCM5 co-operate at the centrioles

It is clear that Tiam1 and MCM5 interact and both proteins are involved in the same regulatory pathway – preventing centriole re-duplication. Given the similar roles of each protein we hypothesise that the interaction between Tiam1 and MCM5 is involved in the regulation of centriole re-duplication. The localisation of MCM5 at the centrosome is important for preventing centrosome re-duplication and previous data has shown that Tiam1 localises at the centrosomes to facilitate centrosome separation (Ferguson and Maller, 2008; Whalley et al., 2015; Woodcock et al., 2010). Therefore Tiam1 and MCM5 may co-localise at the centrosomes to regulate centriole re-duplication.

5.3.5a Tiam1 and MCM5 localisation

Tiam1 and MCM5 were found to co-localise at the centrioles in U2OS tumour cells. Tiam1 behaves like a PCM marker while MCM5 localises distinctly with the centrioles. As Tiam1 is a large scaffolding protein, Tiam1 may be incorporated within the PCM and this might hold MCM5 in place at the centrioles. Pericentrin has been documented to have a similar role within the centrosome. Pericentrin is a large scaffolding protein that anchors multiple centrosomal components to the centrosome – regulating centrosome function (Delaval and Doxsey, 2010). The association of MCM5 at the centrioles, mediated by Tiam1, may in turn block centriole re-duplication. Notably these localisation experiments were done using standard confocal microscopy. Replicating these experiments using high resolution gSTED microscopy would provide extra detail in to the relationship between Tiam1 and MCM5 at the centrioles. Investigating the relationship of Tiam1 and MCM5 with additional centriole/centrosome markers will further elucidate whether Tiam1 is preferentially associated with the PCM and MCM5 is predominantly associated with the centrioles.

Since Tiam1 and MCM5 are important for preventing centriole re-duplication it is anticipated that their restrictive activity will oscillate throughout the cell cycle. There will be a period prior to the initiation of centriole duplication where the suppressive activity of these proteins is reduced to allow centriole duplication. For example, Cep76 has been shown to suppress centriole re-duplication – similarly to Tiam1 and MCM5 (Tsang et al., 2009). The expression of Cep76 is up-regulated during S-phase – coinciding with the completion of normal centriole duplication (Tsang et al., 2009).
Therefore Cep76 expression is coupled with the requirement to block additional duplication events. It is unclear whether Tiam1 and MCM5 show a similar cell cycle dependent association with the centrioles. We found that Tiam1 and MCM5 localise to the centrioles in G1 phase of the cell cycle – a period where the centrioles are disengaged and primed for centriole duplication (Nigg and Stearns, 2011). However G1 phase was based on the interpretation of centriole number – this does not determine whether the cell is in early or late G1. Centriole biogenesis is initiated at the G1/S-phase boundary therefore there may be a period in the early stages of G1 that Tiam1 and MCM5 are absent from the centrioles – permitting the initiation of one round of centriole duplication. Synchronisation experiments should be carried out to determine whether there is a period of the cell cycle where Tiam1 and MCM5 are absent from the centrioles. Protein levels could also be assessed from centrosome fractionations prepared from each cell cycle phase. This would determine whether there is a change in abundance of Tiam1 and MCM5 at the centrioles throughout the cell cycle.

We have also shown that over-expression of Tiam1 has no effect on normal centriole biogenesis. Therefore it is possible that Tiam1 remains at the centrioles throughout the cell cycle and become post-translationally modified following the completion of normal centriole biogenesis. This modification could be important for preventing centriole re-duplication. Interestingly there is a re-distribution of MCM5 protein at the centrioles during mitosis. Throughout interphase MCM5 is associated with a single centriole within a pair whilst during mitosis MCM5 is localised to each centriole within a pair. We know that Tiam1 is phosphorylated by CDK1 during mitosis (Whalley et al., 2015). CDK1 activation is regulated by PLK1 – the initiator of centriole disengagement (Seki et al., 2008; Tsou et al., 2009). Therefore phosphorylation of Tiam1 by CDK1 could cause a conformational change within the protein that re-distributes MCM5 between both centrioles and this re-distribution could be important for centriole disengagement. This again may support the idea of multiple MCM5 binding sites within Tiam1 (Figure 52).
Figure 52. Model 3 - Tiam1 redistributes MCM5 at the centrioles. MCM5 is associated with a single site on the centriole – potentially maintaining the physical interaction between the mother and daughter. A conformational change within Tiam1 during mitosis may cause MCM5 to redistribute between each centriole and this could influence centriole disengagement.

5.3.5b Determining whether Tiam1 knockdown has an effect on MCM5 localisation

We have shown that knockdown of Tiam1 causes the assembly of aberrant centriole structures and that MCM5 is a novel interactor of Tiam1. We hypothesise that this interaction is important for preventing centriole re-duplication therefore knockdown of Tiam1 may affect the localisation of MCM5 at the centrioles – permitting the assembly of aberrant centriole structures. To test our hypothesis we planned to knockdown Tiam1 and assess whether MCM5 is still present at the centrioles. However it was experimentally challenging to eradicate all detectable endogenous levels of Tiam1 for use in IF. This is a problem that has been encountered frequently within the laboratory. siRNA knockdown is not 100% penetrant – some cells will retain expression of Tiam1 and this could interfere with the quantification of MCM5 at the centrioles. Therefore we decided to use CRISPR technology to generate a Tiam1 knockout cell line that can inducibly express Tiam1 WT following the addition of DOX. Unfortunately these cells were heterozygous at the Tiam1 gene locus, providing the same technical issue as using siRNA – there is still endogenous Tiam1 left in the system. Over-expression of Tiam1 WT in these cells caused a modest but not significant increase in MCM5 protein at the centrioles. Therefore it is not clear at this time whether Tiam1 is responsible for the recruitment of MCM5 to the centrioles.
However this experiment can be adapted in several ways to finally determine whether Tiam1 knockdown has an effect on MCM5 protein at the centrioles:

- MCM5 localisation should be assessed in a total Tiam1 knockout cell line – these cells are currently being made. Furthermore a more robust method could be used to quantify levels of Tiam1 and MCM5 at the centrioles as quantification of protein levels by IF is challenging. Centrosome fractionation would allow the analysis of Tiam1 and MCM5 protein levels by western blot.

- We have suggested that Tiam1 and MCM5 may regulate centriole duplication in a complex with Cyclin E. Therefore it is possible that some MCM5 is still recruited to the centrioles by Cyclin E in Tiam1 knockdown cells (Figure 51). This reduction causes a phenotype (increase in centriole number) however there may only be a modest change in MCM5 protein levels at the centrioles. Therefore if it is confirmed that Tiam1-MCM5-Cyclin E co-operate in a complex, MCM5 localisation should be examined in cells that are knockout for both Tiam1 and Cyclin E.

- We have also suggested that Tiam1 is permanently incorporated within the PCM and is responsible for anchoring MCM5 to the centrioles. Therefore knockdown of Tiam1 may not prevent the recruitment of MCM5 to the centrioles, instead Tiam1 knockdown could prevent MCM5 from being retained at the centrioles. This could explain why there is only a modest increase in the localisation of MCM5 at the centrioles following over-expression of Tiam1. To confirm this hypothesis this would require tracking MCM5 protein stability at the centrioles following knockdown of Tiam1 – experimentally this is likely to be challenging.

**5.3.6 Conclusion**

This data strongly suggests that MCM5 is a *bona fide* interactor of Tiam1. Furthermore both of these proteins are localised at the centrioles and both proteins act within the same regulatory pathway – preventing centriole re-duplication (Ferguson and Maller, 2008). Therefore it is likely that the interaction between Tiam1 and MCM5 is important for preventing centriole re-duplication. Further investigation is required to define the full significance of this interaction. However given the preliminary data presented in this chapter Tiam1 may be responsible for the recruitment/anchoring of MCM5 at the centrioles, in turn preventing centriole re-duplication.
Chapter 6. Concluding Remarks

6.1 Tiam1 is a novel regulator of the centriole cycle

The data presented in this thesis has identified Tiam1 as a novel regulator of the centriole cycle. We have shown that Tiam1 prevents centriole re-duplication – independently of Rac activation, and knockdown of Tiam1 is sufficient to increase centriole number in human tumour cell lines. We have identified MCM5 as a novel interactor of Tiam1 and it is clear that both Tiam1 and MCM5 are implicated in the same biological process – limiting centriole re-duplication (Ferguson and Maller, 2008). However the full biological significance of this interaction remains unclear and at this stage we are unable to describe mechanistically how Tiam1 and MCM5 co-operate to prevent promiscuous duplication events. However given the data presented in this thesis we can propose the following general mechanism – Tiam1 and MCM5 are involved in maintaining the physical interaction between the mother and daughter centrioles (the S-M linker). Knockdown of Tiam1 compromises the integrity of this interaction, promoting premature disengagement and centriole amplification (Figure 53).

Figure 53. Tiam1 co-operates with MCM5 at the centrioles to maintain the S-M linker. Tiam1 knockdown compromises the activity of MCM5 at the centrioles licensing centriole disengagement. This allows the centrioles to duplicate multiple times within the same cell cycle.
6.2 Regulation of centriole number by Tiam1 in vivo

Numerous studies implicate Tiam1 in tumourigenesis (Mack et al., 2011). Total knockout of Tiam1 was found to have a protective effect in mice treated with a Ras-induced skin carcinogenesis protocol (Malliri et al., 2002). Tiam1 knockout mice produced significantly less skin tumours when compared to wild type (Malliri et al., 2002). However a small number of tumours did develop in the Tiam1 knockout mice and significantly these tumours were more likely to progress to malignancy (Malliri et al., 2002). This could be due to the established role of Tiam1-mediated Rac signalling in the maintenance of cell-cell adhesions (Woodcock et al., 2009; Vaughan et al., 2015). However this thesis has identified Tiam1 as a novel regulator of the centriole cycle. Knockout of Tiam1 in vivo may promote centriole amplification and lagging chromosomes at anaphase – promoting CIN. This may contribute to the malignant phenotype observed in Tiam1 knockout tumours. Therefore it will be extremely interesting to determine whether loss of Tiam1 is sufficient to promote centriole amplification in tumours derived from the Tiam1 knockout mouse.

6.3 The therapeutic potential of Tiam1

Aberrations in centrosome number are common in human tumour cells and multiple studies are beginning to address the biological consequence of centrosome amplification in cancer (Godinho and Pellman, 2014; Nigg, 2002; Zyss and Gergely, 2009). Several lines of evidence suggest that centrosome amplification correlates with tumour aggressiveness and poor patient outcome (Chan, 2011; Lingle and Salisbury, 2000; Landen et al., 2007). As centrosome number is dictated by the centrioles it is crucial to understand the mechanisms that regulate centriole number and how these processes become de-regulated in cancer. Characterisation of proteins involved in the regulation of centriole number could identify new markers that could identify tumours with centrosome amplification, identifying patients that would benefit from treatment with centrosome de-clustering drugs.

We have identified Tiam1 as a novel regulator of centriole number – specifically regulating centriole re-duplication. Loss of Tiam1 was sufficient to promote the assembly of aberrant centriole structures in human tumour cell lines. Therefore Tiam1 has the potential to act as a prognostic marker for centriole amplification.
Tumours showing undetectable or low expression of Tiam1 could be targeted with centrosome de-clustering agents such as HSET. This would selectively target tumour cells with supernumerary centrioles, promoting multipolar spindle formation and cell death (Watts et al., 2013). However the role of Tiam1 in the centriole cycle must be investigated in vivo. We suspect that loss of Tiam1 may permit the assembly of aberrant centriole structures in cells that are already amenable to centriole amplification – ie. they have an underlying cell cycle defect caused as a result of malignant transformation. It would be interesting to determine whether Tiam1 knockdown has an effect on centriole number in a transformed in vivo background.

6.4 Key findings

The data presented in this thesis has provided strong evidence that Tiam1 is a novel regulator of the centriole cycle – Tiam1 prevents centriole re-duplication and this is independent of the downstream activation of Rac. Supernumerary centrioles in Tiam1 knockdown cells resemble fully functional centrioles and are capable of contributing to spindle assembly. Furthermore the assembly of aberrant centrioles correlates with an increased frequency of lagging chromosomes during anaphase. This suggests that aberrant centriole structures in Tiam1 knockdown cells can interfere with bipolar spindle assembly, promoting chromosome segregation errors and CIN. MCM5 is a novel Tiam1 interactor and Tiam1 co-operates with MCM5 to prevent centriole re-duplication, potentially by maintaining the integrity of the S-M linker. With further investigation Tiam1 could be used as a marker to identify patients with tumours that have centrosome amplification and CIN – a factor that predates poor patient prognosis. This could identify a cohort of patients that would benefit from treatment with centrosome de-clustering drugs
Appendix

Appendix Table 1. Tiam1 peptide sequences.

<table>
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<tr>
<th>Peptide Number</th>
<th>Amino Acid Sequence</th>
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<tr>
<td>11</td>
<td>T-R-S-S-S-T-P-S-I-P-Q-S-L-A-E-N-G-L-E-P-F-S-Q-D-G</td>
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<tr>
<td>14</td>
<td>N-G-L-E-P-F-S-Q-D-G-T-L-E-D-F-G-S-P-I-W</td>
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<td>15</td>
<td>F-S-Q-D-G-T-L-E-D-F-G-S-P-I-W-V-D-R-V-D</td>
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<td>16</td>
<td>T-L-E-D-F-G-S-P-I-W-V-D-R-V-D-M-G-L-R-P</td>
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<td>187</td>
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Total: 173
Appendix Figure 1. The Tiam1 peptide array. Arrangement of Tiam1 peptides on the array. Numbers correspond to peptide sequences shown in Appendix Table 1.
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