The role of phosphorylation of TRAPPC12 in mitosis

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ABSTRACT

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Transport Protein Particle (TRAPP) is a multisubunit tethering complex that is involved in membrane trafficking. Lately, there have been numerous cases of proteins with membrane trafficking functions that have been found to have separate functions. These moonlighting functions occur either in a distinct cellular location or during a different phase of the cell cycle. Here we present the unexpected finding that a protein TRAPPC12, a subunit of the mammalian TRAPPIII membrane trafficking complex, plays a role in mitosis. Depletion of TRAPPC12 results in mitotic arrest of the cells. Our results show that TRAPPC12 is hyperphosphorylated early in mitosis and dephosphorylation of TRAPPC12 is necessary for the cell cycle progression. Finally, we demonstrate that a phosphomimetic form of TRAPPC12 leads to an increase in mean mitotic index than does the non-phosphorylatable mutant. Our study identifies a moonlighting function in mitosis for TRAPPC12 that it is regulated by its phosphorylation and dephosphorylation.

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Table of Contents

List of Tables

List of Figures

List of Acronyms

APC/C: Anaphase promoting complex/cyclosome

ATM: Ataxia telangiectasia mutated

ATP: Adenosine 5'-triphosphate

ATR: ataxia telangiectasia and Rad3-related protein

BSA: Bovine Serum Albumin

Bub: Budding uninhibited by benzimidazole

BubR1: Bub1-related kinase 1

CCAN: constitutive centromere-associated network

Cdc20/25B: Cell division cycle 20/25B

CDK: cyclin dependent kinase

CENP: Centromeric protein

COPI/II: coat protein complex I/II

CPC: Chromosomal passenger complex

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DNA: deoxyribonucleic acid

DTT: dithiothreitol

EDTA: ethylenediaminetetraacetic acid

Eg5: Kinesin family member 2B

ER: endoplasmic reticulum

ERGIC: ER-Golgi intermediate compartment

FBS: fetal bovine serum

G1: Gap 1

G2: Gap 2

Gadd45: growth arrest and DNA damage inducible gene

GEF: guanine exchange factor

GRASP65: Golgi reassembly stacking protein of 65 kDa

Hec1/Ndc80: Highly expressed in cancer1/kinetochore protein Ndc80 homologue

HeLa: Human cervical carcinoma cell line

H3: Histone H3

kDa: kilo Dalton

KMN: KNL-1/Mis12 complex/NDC80

Knl1: Kinetochore null protein 1

LB: Luria broth

M: Mitosis

Mad: Mitotic arrest deficient

Mad2: Mitotic arrest deficient 2-like protein 1

MCC: Mitotic checkpoint complex

MT: microtubules

NEBD: Nuclear envelope breakdown

PAGE: polyacrylamide gel electrophoresis

PBD: Polo-box domain

PBS: phosphate buffered saline

PBSt: PBS tween

PCR: polymerase chain reaction

Plk: Polo – like kinase

S phase: synthesis phase

SDS: sodium dodecyl sulphate

siRNA: small interfering ribonucleic acid

TRAPP: transport protein particle

1. INTRODUCTION

1.1 The cell cycle

In 1882, Walther Flemming was one of the first scientists to present a detailed description of the events that take place during cell division in animals and named the division of somatic cells “mitosis” (Flemming, 1882, see the translation in 1965). He originally coined the term “mitosis” from the Greek word for thread, because of the shape of mitotic chromosomes. The precision and fidelity of a cell to divide into two daughter cells that contain identical genetic material is vital for all forms of life. The event that leads to the reproduction of all cells is called the cell cycle. In eukaryotic cells the cycle is composed of five distinct phases: G1 (Gap1), S (Synthesis)-phase, G2 (Gap2) (collectively referred to as interphase), Mitosis and Cytokinesis (collectively referred to as M-phase). Mitosis can be further subdivided into a number of distinct phases that are distinguished both morphologically and biochemically.

1.1.1 Interphase

Cells spend most of their time in interphase where they are metabolically active and growing, preparing for cell division. Interphase is divided into three phases: G_1 (Gap 1), where the cell grows and prepares for entry into S (DNA synthesis) phase; S-phase, which is characterized by replication of DNA; and G_2 (Gap 2) phase which ensures that the DNA is correctly replicated before the cell irreversibly enters mitosis (Blow and Tanaka, 2005; Nurse, 2001).

1.1.2 Mitosis

One recognizable feature of a eukaryotic cell is the presence of the nucleus that contains the genetic material. After the DNA is duplicated in S phase the cells prepare to

enter mitosis where duplicated DNA condenses into chromosomes (organized structures of DNA and protein). Chromosomes are composed of two sister chromatids (identical copies of a chromosome), each of which will be segregated into a different daughter cell. Mitosis can be divided into five progressive phases called prophase, prometaphase, metaphase, anaphase and telophase (Moorhead and Hsu, 1956) that work together through different checkpoints to ensure the equal distribution of the sister chromatids to each daughter cell. Cytokinesis begins at the same time as telophase and often is thought of as a part of it, but it is indeed a separate process (Figure 1.1).

In most eukaryotes the nuclear envelope breaks down during prophase. During prophase, the paired chromosomes condense and are bound together at the centromere by the cohesin complex (Lloyd and Chan, 2006). At this time, key complexes and proteins such as the Ndc80 complex, KNL and the Mis12 complex start to bind to the kinetochore (a larger structure at the centromeric region of the chromosomes; see below section 1.4) preparing for spindle microtubule attachment. This is followed by prometaphase where microtubules elongate from centrosomes capturing the newly replicated chromosomes which then congress to the metaphase plate (Kotwaliwale and Biggins, 2006) where they align during metaphase (Maiato et al., 2004). Anaphase begins with the separation of sister chromatids that start moving to opposite poles of the cell due to shortening of kinetochore microtubules. Afterward, the polar microtubules elongate and the two poles of the spindle move farther apart. The nuclear envelope starts to reform around the decondensed chromosomes and metabolic activity is restored during telophase. The separation of cytoplasm and the formation of the cleavage furrow ingression occurs during cytokinesis, leading to the formation of two daughter cells (Glotzer 2005).

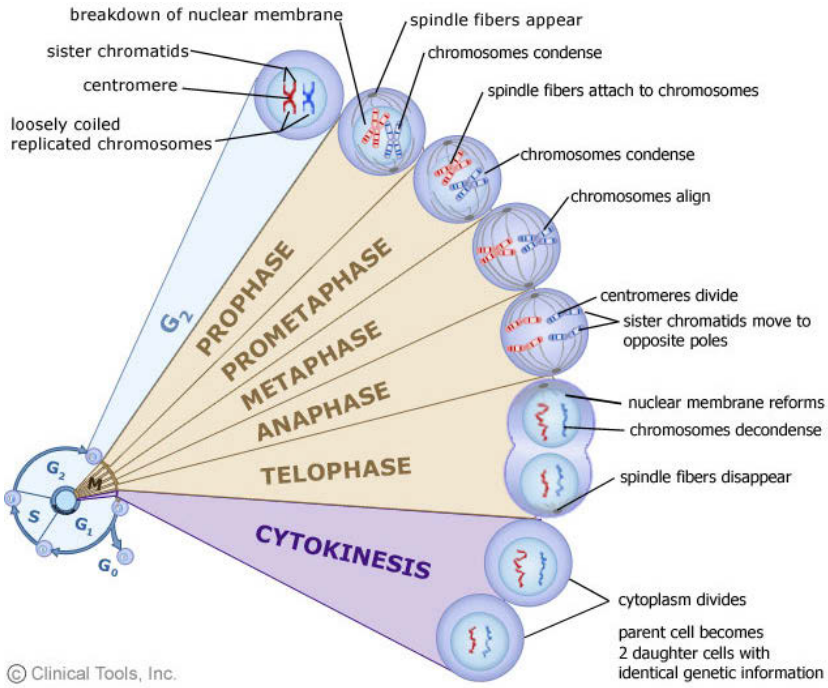

Figure 1.1. An overview of Mitosis. During early mitosis, the pairs of sister chromatids are attached to the mitotic spindle by microtubules. By mid-mitosis or metaphase, each of the pair of sister chromatids are attached to separate microtubules, originating from opposite poles of the spindle, with sister chromatid cohesin destroyed during anaphase, whereby microtubules pull the separated chromosomes to opposite ends of the cell. The two sets of chromosomes are packaged into two daughter nuclei, with constriction of the cytoplasm and deposition of a new membrane during telophase/cytokinesis marking the end of the cell cycle. Image taken from: http://www.le.ac.uk/ge/genie/vgec/he/cellcycle.html.

1.2 Mitotic kinases

Phosphorylation is a major posttranslational modification of proteins involved in many stages of the cell cycle, especially during mitosis. Protein phosphorylation is mediated by kinases, some of which are critical to the progression of mitosis. The most crucial kinases involved in mitosis will be discussed in the sections below.

1.2.1 Cyclin-dependent kinases

Cyclin-dependent kinases (CDKs), a family of multifunctional enzymes, are among the main factors that drive cell cycle progression in mammalian cells (Morgan, 2010; Murray, 2004). The enzymatic activity of CDKs varies with expression levels of the cyclins that bind to CDKs and activate them. The formation of cyclin-CDK complexes drives the cell cycle by phosphorylating substrates and changing their enzymatic activity or their interaction with other proteins. The amount of CDKs remains constant during the cell cycle compared to the varying levels of cyclins (Arellano and Moreno, 1997). The production and degradation of specific cyclins during various phases of the cell cycle is what drives the cell cycle from one phase to another, and these cyclins can be used as biochemical markers for stages of the cell cycle. Failure to degrade specific cyclins results in the arrest of the cell cycle (Fisher et al., 2012). In the beginning of G1, expression of cyclin D activates CDK4 and CDK6, and cyclin E binds to CDK2 (Shapiro, 2006). During S phase cyclin A activates CDK2 and has an important role in DNA replication (De Boer et al., 2008). In early G2 phase, cyclin A associates with CDK1 driving the progression of the cell through G2 phase (De Boer et al., 2008; DeGregori and Johnson, 2006; van den Heuvel and Dyson, 2008; Vigo et al., 1999). Cyclin B complexed with CDK1 is the primary CDK complex required for mitotic progression (Arellano and Moreno, 1997). The levels of cyclin B start increasing during G2 phase when it binds to CDK1 but the complex is kept inactive at this stage by phosphorylation

of the T14 and Y15 residues of CDK1 by the Wee and Myt kinases (Mueller et al., 1995; Parker and Piwnica-Worms, 1992) and is activated in prophase by dephosphorylation of these sites mediated by Cdc25 phosphatase (Boutros et al., 2006). While cyclin B-CDK1 is mainly activated within the nucleus, cyclin B contains a nuclear export signal (Moore et al., 1999) which localizes it to the cytoplasm until the beginning of prophase (Porter and Donoghue, 2003). Therefore, the initial activation of cyclin B-CDK1 occurs in the cytoplasm, which controls the timing of its activation in the nucleus. Cyclin B-CDK1 is imported into the nucleus just before nuclear envelope breakdown (NEBD) (Gavet and Pines, 2010; Peter et al., 1991). The cyclin B-CDK1 complex is known to phosphorylate a number of substrates during mitosis and, thus, it is involved in different functions such as chromosome condensation, centrosome separation, and assembly of the mitotic spindle (Blangy et al., 1995; Kimura, Hirano, Kobayashi and Hirano, 1998). It is also involved in the regulation of the anaphase- promoting complex/cyclosome (APC/C) (Sudakin et al., 1995; Zachariae et al., 1998).

1.2.2 Polo-like kinases

Polo-like kinases (PLKs) also have an essential role in the progression of the cell cycle. All PLKs have an N-terminal serine/threonine kinase domain, a highly conserved C-terminal polo-box domain (PBD) and are activated by phosphorylation of a region called the T-loop (Mundt et al., 1997). It has been shown that PLKs cooperate with CDKs to regulate the cell cycle through an involvement in the activation of cyclin B-CDK1 during mitotic entry (Toyoshima-Morimoto at al., 2002; Watanabe et al., 2004). Plk1 is a member of the PLK family that is highly expressed during mitosis (Barr et al., 2004). The activity of Plk1 is regulated by phosphorylation (Barr et al., 2004) and protein degradation (Lindon and Pines, 2004). During mitosis, Plk1 is located in various subcellular structures including centrosomes, kinetochores, the central spindle and the

midbody, demonstrating the critical role that this kinase has in the orchestration of cell division (Arnaud at al., 1998; Golsteyn et al., 1995; Petronczki et al., 2008).

1.2.3 Aurora kinases

Aurora kinases have been found to play an essential role in different mitotic events. The Aurora family components are serine/threonine kinases that regulate essential processes from mitotic entry to cytokinesis (Dutertre et al. 2002). In mammals, three members of the Aurora kinase family are known: Aurora A, B and C. Aurora A is mainly involved in centrosome maturation and the separation of the duplicated centrosomes (Terada et al. 2003; Mori et al. 2007; Glover et al. 1995; Liu and Ruderman 2006). Aurora A kinase involvement is also required for the activation of Cdc25B at the centrosomes that leads to initial cyclin B-CDK1 activation at the centrosomes during the G2/M transition (Cazales et al. 2005, Lindqvist et al. 2005, Seki et al. 2008).
Aurora B has a broader function in mitotic events and is the catalytically active member of the chromosomal passenger complex (CPC), a central regulator of mitotic progression (Ditchfield et al., 2003; Hauf et al., 2003). An essential role of Aurora B is the correction of kinetochore-microtubule attachment by phosphorylating key substrates at the kinetochore (see below, section 1.4) such as CENP-A and the chromosome passenger complex (CPC) subunits survivin, borealin and inner centromere protein (INCENP). Aurora B promotes the destabilization of abnormal chromosome attachments and kinetochore-microtubule turnover in merotelic (a single kinetochore is attached to microtubules emanating from both spindle poles) attachments (Lampson et al., 2004, Cimini et al., 2006). This leads to an “indirect” role in the activation of the spindle assembly checkpoint (SAC, see section 1.3.2) that results in the inhibition of the anaphase-promoting complex (APC/C). Aurora B also senses the tension at the kinetochore-centromere region leading to SAC activation in response to the absence of

tension (Cimini, 2007; Musacchio and Salmon, 2007). In contrast, little is known about Aurora C but it has been shown that its function overlap and is able to compensate for the loss of Aurora B. This may be due to sequence homology between Aurora B and C in the central catalytic kinase domains (Nigg, 2001; Sasai et al., 2004).

1.3 Cell cycle checkpoints

Checkpoints are control mechanisms that monitor the cell cycle, effectively arresting cell cycle progression at various steps until previous events has been successfully completed. The central components of the cell cycle control are cyclin-dependent kinases (CDKs) (see section 1.2.1). Errors during cell division might lead to serious diseases such as cancer, cardiovascular disease, autoimmune and metabolic disorders, viral infections, atherosclerosis and premature aging (Bicknell and Brooks, 2008; Foster, 2008; Zhivotovsky and Orrenius, 2010). In order to avoid cell division errors, the cell verifies that proper conditions are satisfied at crucial steps in the division process. In general, cell cycle progression is governed by three major checkpoints: G1/S checkpoint, G2/M checkpoint and the mitotic Spindle Assembly Checkpoint (SAC).

1.3.1 G1 and G2 Checkpoints

The presence of DNA damage is a common factor that triggers the activation of both G1 and G2 checkpoints. There are different types of DNA damage that lead to checkpoint arrest. For example the damage that results in single stranded breaks caused by external stimuli activates the ATR dependent pathways and other damages that result in double stranded breaks activate the ATM protein kinase activity (Chan et al., 2000).

The G1/S checkpoint occurs during G1 phase, and it monitors nutritional status, cell size, DNA/cellular damage and growth factor availability. If this checkpoint is activated in G1, it will delay the onset of S-phase and DNA replication (Stein et al., 2012). The G1/S checkpoint is dependent upon the tumour suppressor gene, p53, which protects cells from becoming tumorigenic (Vogelstein et al., 2000). ATM and ATR are involved in sensing DNA damage and can activate p53 via phosphorylation during the G1/S checkpoint (el-Deiry et al., 1993).

The G2/M checkpoint occurs during G2-phase, and it monitors the completion of DNA replication, assuring correct transmission of genetic material (Stein et al., 2012).The G2 phase DNA damage checkpoint has both p53 dependent and independent mechanisms (Chang and Eastman, 2012; Taylor and Stark, 2001). The p53-dependent arrest activates a long term arrest (days), whereas p53-independent mechanisms initiate an immediate arrest that can be stable for a matter of hours (Bunz et al., 1998). Both mechanisms target the regulation of the cyclin B-CDK1 complex, required for M phase progression, however the mechanisms by which these are imposed are different.

p53-independent mechanisms involve the kinases, ATR/ATM, which through their downstream targets and subsequent phosphorylation cascades lead to cyclin B-CDK1 inactivation. ATM/ATR initiates a more rapid checkpoint response to DNA damage than p53, however if a prolonged arrest is required p53 dependent pathways will predominate. This may be facilitated by ATM phosphorylation of the p53 inhibitor, MDM2, as well as ATM/ATR phosphorylation of p53 itself on Ser15 to increase p53 stability (Canman et al., 1998; Khosravi et al., 1999; Maya et al., 2001; Tibbetts et al.,

1999). Therefore the pathways activated during the G2 checkpoint are dependent on the extent of damage.

1.3.2 The Spindle Assembly Checkpoint

The spindle assembly checkpoint (SAC) is an important mitotic control that ensures equal chromosome segregation by suspending division until all chromosomes are oriented correctly at the mitotic spindle. The core SAC proteins (Minshull et al., 1994) include the MAD proteins Mad1, Mad2, and Mad3 (BubR1 in humans) (Li and Murray, 1991) and the BUB proteins Bub1 and Bub3 (Hoyt et al., 1991), all of which are conserved among eukaryotes.

The proper segregation of chromosomes during anaphase is a crucial step of mitosis. The SAC guards the fidelity of chromosome segregation by preventing the cell from entering anaphase until all chromosomes are attached to the spindle apparatus in a bipolar (sister kinetochores are attach to microtubules extending from opposite spindle poles) manner and that the proper tension is applied across the sister centromeres (Musacchio and Salmon, 2007). Incorrect chromosome segregation may lead to cell death (Weaver and Cleveland, 2005) or generate aneuploidy (Kim and Kao, 2005; Steuerwald, 2005), a condition where there are unequal numbers of chromosomes in the daughter cells. The SAC delays anaphase in the presence of unattached or incorrectly attached kinetochores by preventing the activation of the anaphase-promoting complex (APC/C). The anaphase-promoting complex (APC) contains a ubiquitin ligase also known as the cyclosome and it is activated by a cofactor called Cdc20. The SAC negatively regulates Cdc20 which in turn inhibits the activation of APC-mediated polyubiquitylation of two key substrates: Cyclin B1 and securin (Glotzer et al. 1991). By preventing the degradation of cyclin B1 the cell cannot exit mitosis (see section 1.2.1).

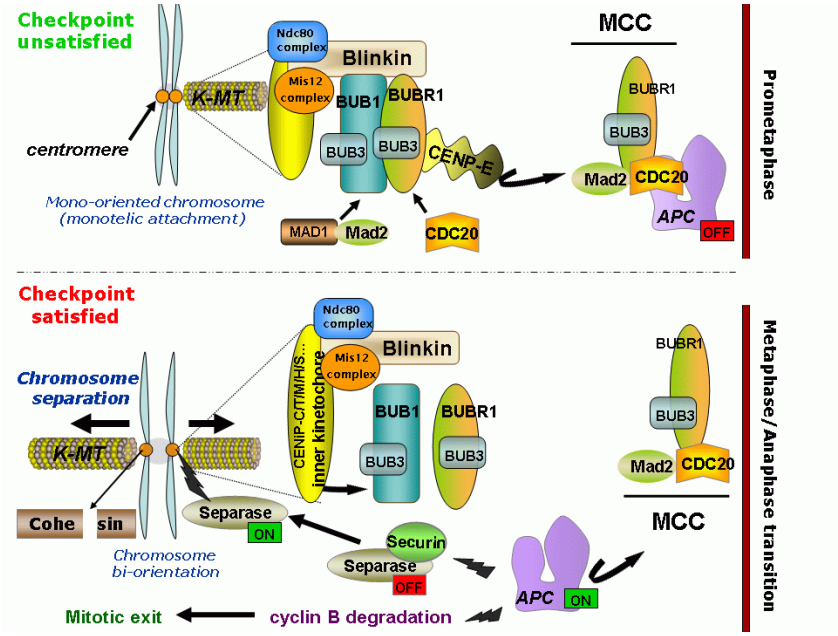

Figure 1.2. Principles of the mitotic SAC. Unattached kinetochores catalyze formation of the mitotic checkpoint complex (MCC) composed of BubR1, Mad2, Cdc20 and Bub3, which block the APC/C. Once all the chromosomes are aligned with their kinetochores attached to the spindle (metaphase), the MCC disassembles, releasing Cdc20, a cofactor of APC/C. Activated APC/C ubiquitylates its substrates, securin and Cyclin B1, which are degraded by the proteasome leading to release of separase and Cdk1. Separase cleaves cohesin allowing sister chromatids to separate, which marks the onset of anaphase. Image taken from: http://www.le.ac.uk/ge/genie/vgec/he/cellcycle.html

Polyubiquitylation of securin is important for full activation of an enzyme called separase that specifically cleaves the cohesion complex that holds the sister chromatids together. (Figure 1.2) (Nasmyth, 2001).

The mechanism of the inhibition of APC/C by the SAC is still unclear. It may be due to the binding of Cdc20 to SAC components or due to the fast turnover of SAC components at unattached kinetochores (Musacchio and Salmon, 2007). Several studies have proposed a “two step” model for activation and maintenance of the SAC. The first is the kinetochore-independent step where the APC/C is inhibited by the production of the mitotic checkpoint complex (MCC), a cytosolic multisubunit complex composed of Mad2, BubR1 and Bub3, and also by the APC/C regulator Cdc20. The MCC is found to be present and active in interphase cells so its inhibition of APC/C activity starts early in mitosis, before kinetochore assembly.

The second step is kinetochore-dependent and starts in prophase where SAC proteins accumulate at unattached or incorrectly attached kinetochores, and Cdc20 is seized by Mad2 and BubR1 (Ciliberto and Shah, 2009; Musacchio and Salmon, 2007). When all kinetochores are correctly attached and aligned at the metaphase plate and under tension, Cdc20 dissociates from the complex (Mad2-Cdc20 and Bub3-BubR1-Cdc20) and activates the APC/C. In turn, activation of APC/C mediates degradation of both cyclin B1 and securin, and eventual mitotic exit.

1.4 Kinetochores

Accurate chromosome segregation is achieved when all of the chromosomes are oriented correctly at the metaphase plate. Kinetochores are specialized macromolecular protein structures that aid in chromosome movement to the metaphase plate.

Kinetochores assemble at the centromere of each sister chromatid and interact with spindle microtubules during mitosis (Maiato et al., 2004). This interaction creates a tension on the bioriented kinetochores that is caused by the pulling force of spindles on kinetochore microtubules countering the cohesion of the sister chromatids. In the past few years there has been an exponential increase in the identification and understanding of the proteins localized at the kinetochore, although more is left to be discovered (Cheeseman and Desai 2008; Chan et al. 2005; Santaguida and Musacchio 2009). Genomic and proteomic studies have revealed that the kinetochore may exceed 200 proteins (Ohta et al., 2010; Tipton et al., 2012). In mammalian cells kinetochores are divided into two layers called the inner and outer kinetochore. The inner kinetochore is located adjacent to the centromere and contains approximately 100 proteins including the histone H3 variant CENP-A (Fachinetti et al., 2013). The outer kinetochore contains roughly 20 known proteins that assemble adjacent to the inner kinetochore after nuclear envelope breakdown (NEBD). Most of the known microtubule interacting proteins like CENP-E, dynein and checkpoint proteins are part of the outer kinetochore (Cooke et al., 1997a). The outer kinetochore proteins are divided into subgroups that localize to the kinetochore at different points of mitotic progression and their localization was determined based on many experiments and assays throughout the years (Cheeseman et al.i, 2006; Cheeseman et al., 2008; Foltz et al., 2006; Liu et al., 2006; Maiato et al., 2004; Musacchio and Salmon, 2007). Three important complexes of the outer layer of the kinetochore are the Mis12 complex, Knl1 and the Ndc80 complex (collectively referred to as the KMN network) which serve as a scaffold for SAC proteins but are also key regulators of microtubule attachment (Cheeseman et al., 2006). The association of the KMN network with kinetochores begins in prophase and ends in telophase (Santaguida and Musacchio, 2009). The Ndc80 complex consists of four members including Ndc80, (Hec1 in humans), Nuf2, Spc24 and Spc25, and it is essential for

kinetochore-microtubule attachments (DeLuca et al., 2002; McCleland et al., 2003). The Mis12 complex is composed of Nnf1, Mis12, Dsn1 and Nsl1 and it serves as a major platform for outer kinetochore assembly (Cheeseman and Desai, 2008). Knl1 (also referred as CASC5 or blinkin in humans) is recruited to the kinetochore by Mis12 complex and it has been shown to have a role in the recruitment of other outer kinetochore proteins such as ZWINT and CENP-F, and SAC proteins such as BUB1 and BUBR1 (Cheeseman et al., 2008). The microtubule binding affinity of the kinetochores is increased when the Ndc80 complex associates with Knl1 and Mis12 complex (Cheeseman et al., 2006). Preventing one of the KMN members from localizing to the kinetochore results in chromosome segregation defects and either a partial or complete detachment of the kinetochore from the microtubules (Cheeseman et al., 2008; DeLuca et al., 2005; Kline et al., 2006). Phosphorylation of KMN proteins by Aurora B kinase decreases their affinity for microtubules. When the microtubule is properly attached to the kinetochore, the increased tension pulls the KMN network further away from Aurora B, thereby decreasing the ability of Aurora B to phosphorylate the KMN network. In the case of incorrectly attached microtubules, the KMN network cannot be pulled away from Aurora B. Keeping the KMN network phosphorylated leads to destabilized microtubule-kinetochore attachments that allow the microtubule to detach and try again.(Cheeseman et al., 2006).

Assembly of the kinetochore onto centromeric DNA is mediated by the modified histone H3 variant called CENP-A that is part of the inner kinetochore. CENP-A is part of a network of 16 peripheral centromeric proteins including CENP-C, -H, -I, -K, -L, -M, -N, -O, -P, -Q, -S, -T, -U, -W and –X that is collectively referred to as the constitutive centromere-associated network (CCAN) (Foltz et al., 2006; Hori et al., 2008; Okada et al., 2006). The main function of the CCAN is to link the centromeric DNA on the inner

kinetochore with the microtubule-bound KMN network of the outer kinetochore (Hori et al., 2008; see review by Varma and Salmon, 2012).

Spindle microtubules that attach to kinetochores are called kinetochore microtubules. Kinetochores have multiple sites where the microtubules attach by the plus end and as the microtubules shrink the kinetochore (and the chromosome) moves towards the spindle pole where the minus end of the microtubule is attached (Wittmann et al., 2001).

1.5 Motor proteins

Motor proteins use the energy from ATP hydrolysis to regulate microtubule dynamics, help to perform microtubule sliding, move chromosomes along microtubules or pull microtubules toward the cell poles (Zhu et al., 2005). One of the major driving forces of spindle bipolarity is the microtubule sliding and it is mainly carried out by the plus end-directed kinesin-5 Eg5/KIF11 (Blangy et al., 1995; Zhu et al., 2005). Another motor protein is the centromere-associated motor protein CENP-E. CENP-E is implicated in chromosome positioning, it is important for chromosome congression and helps align chromosomes towards the metaphase plate (Cooke et al., 1997a; Kapoor et al., 2006; Schaar et al., 1997; Yen et al., 1991).The essential roles of motor proteins during mitotic spindle assembly make them good molecular targets for anticancer therapy and currently inhibitors of Eg5 and CENP-E are being tested in clinical trials (Rath and Kozielski, 2012).

1.5.1 CENP-E

CENP-E was first discovered in human cells with a monoclonal antibody against human mitotic chromosome scaffold proteins. It was later found to belong to the kinesin

family of motor proteins (Kinesin 7) and localized at the kinetochore (Yen et al., 1991;Yen et al., 1992). CENP-E is a large protein (312 kDa in human) with a kinesin motor domain at the N-terminal that activates BubR1 kinase activity (Mao et al., 2003). The expression of CENP-E is regulated in a cell cycle-dependent manner that is consistent with its function during mitosis (Weaver et al., 2003). CENP-E is recruited at the kinetochore shortly after NEBD and it localizes to the outer kinetochore from early prometaphase through metaphase. During late anaphase and telophase it moves to the spindle midbody after which it starts to gradually degrade (Cooke et al., 1997b; Yao et al., 1997; Brown et al., 1996). Initially, CENP-E was reported to be a minus end-directed motor (Thrower et al., 1995) but later Wood and co-workers showed the recombinant motor domain to move toward the microtubule plus-end in vitro (Wood et al. 1997).

1.5.1.1 Role of CENP-E in the microtubule capture at kinetochores

Studies have shown that interfering with CENP-E function results in one or a few misaligned chromosomes (Putkey et al., 2002; Mcewen et al., 2001; Yao et al., 2000). CENP-E is responsible for the stable capture of kinetochores by spindle microtubules and is essential for congression of initially unaligned chromosomes (Yen et al., 1991; Kapoor et al., 2006; Schaar et al., 1997). Altering CENP-E function by using antibody injection (Schaar et al., 1997), depletion (Wood et al. 1997, Yao et al., 2000), gene inactivation (Putkey et al., 2002), or inhibition with a small-molecule inhibitor (Wood et al., 2010) blocks microtubule plus-end-directed motion of pole-proximal chromosomes, which is followed by a failure of kinetochores to form stable microtubule attachments. The general assumption that chromosome congression requires all chromosomes to be bioriented before congression was challenged in 2006 by Kapoor and colleagues where they showed that chromosomes can congress to the metaphase plate before biorientation through a mechanism that is dependent upon CENP-E (Kapoor et al.,

2006). In the CENP-E- dependent mechanism, the monooriented chromosome (a chromosome attached only at one of the kinetochores by spindle microtubules) is able to congress to the metaphase plate by using kinetochore fibers of the already bioriented chromosomes. This way, CENP-E plays an important role in transporting the monooriented chromosomes from the spindle pole to the spindle plate where they can find connections from the opposite pole and become bioriented (Kapoor et al., 2006).

1.5.1.2 Role of CENP-E in the spindle assembly checkpoint

In addition to its role in kinetochore-microtubule capture, CENP-E has been reported to have a role in the spindle assembly checkpoint. CENP-E's role in the SAC is best described as the binding partner of the checkpoint kinase BubR1 (Chan et al. 1999; Mao et al., 2003b; Yao et al., 2000). Among other proteins of the SAC, CENP-E and BubR1 are two important proteins of the outer kinetochore. BubR1 has a role in kinetochore-microtubule attachment and metaphase chromosome alignment in mammalian cells and in vitro assays of recombinant CENP-E and BubR1 have shown that CENP-E greatly enhances the activity of BubR1 toward itself (Lampson and Kapoor, 2005; Mao et al., 2003b; Weaver et al., 2003). Kinase activity of BubR1 is active in mitotic cells and it is important for its checkpoint function by monitoring CENP-E-dependent activities at the kinetochore and regulating APC/C activity (Chan et al., 1999). In the presence of one or few unattached kinetochores, CENP-E recruits BubR1 to the polar kinetochores leading to the activation of the SAC (Weaver et al., 2003). The checkpoint activity of BubR1 is regulated by conformational changes in CENP-E when it interacts with microtubules, thus implicating CENP-E in the activation and maintenance of mitotic checkpoint signaling (Abrieu et al., 2000). Upon capture of the microtubules, CENP-E silences the BubR1 kinase activity by forming a ternary complex of BubR1-CENP-E-microtubule (Mao et al., 2005). BubR1 in the ternary complex is unable to

associate with Cdc20, which in turn can be free to activate the APC/C complex (see section 1.3.2) (Mao et al., 2003b). Compared with the depletion of Mad2 or BubR1 in human cells, the depletion of CENP-E does not speed up mitosis (Meraldi et al., 2004). Instead it leads to a long term mitotic arrest caused by one or more misaligned chromosomes, indicating that the checkpoint is functional in humans in the absence of CENP-E (Yao et al., 2000). A weak checkpoint does not result in cell death, and interference with essential checkpoint genes in mice was shown to lead to an increase in aneuploidy (Weaver and Cleveland, 2005; Weaver et al., 2003).

1.6 TRAPP complex

Proliferating cells must harmonize their growth and division so that cells divide when they have grown sufficiently. Mitosis and membrane trafficking are among most essential processes in eukaryotic cells. Membrane trafficking allows for cells to maintain cellular homeostasis by delivering new materials to the plasma membrane via the secretory pathway. In the secretory pathway, vesicle-mediated transport between organelles ensures specificity in protein localization. Transport protein particle (TRAPP) is a multisubunit tethering factor that is evolutionarily conserved. Initially, TRAPP was described as a large protein complex that functions in endoplasmic reticulum (ER)-to-Golgi traffic in yeast (Sacher et al., 1998; Sacher et al., 2000). Later, more distinct forms of TRAPP with specific functions were identified. To date, there are three known TRAPP complexes in the budding yeast *Saccharomyces cerevisiae* that function in ER-to-Golgi traffic (TRAPP I) (Sacher et al., 1998), post-Golgi trafficking (TRAPP II) (Sacher et al., 2001) and autophagy (TRAPP III) (Lynch-Day et al., 2010). In contrast, in mammalians only TRAPP II and III have been observed (Bassik et al., 2013). The mammalian TRAPP complex contains homologues of all yeast TRAPP subunits named from TRAPPC1 to TRAPPC10, plus the recently characterized subunits TRAPPC11, TRAPPC12, and

Mammalian TRAPP subunits (kDa)	Accession numbers	Complex
TRAPPC1 (17)	NP_001160093.1	II, III
TRAPPC2L (16)	NP_057293.1	II, III
TRAPPC2 (16)	NP_001122307.2	II, III
TRAPPC3 (20)	NP_001257824.1	II, III
TRAPPC4 (24)	NP_057230.1	II, III
TRAPPC5 (21)	NP_001035927.1	II, III
TRAPPC6a (19)	NP_001257822.1	II, III
TRAPPC6b (15)	NP_001073005.1	II, III
TRAPPC8 (161)	NP_055754.2	III
TRAPPC9 (140)	NP_001153844.1	II
TRAPPC10 (142)	NP_003265.3	II
TRAPPC11 (129)	NP_068761.4	III
TRAPPC12 (79)	NP_057114.5	III
TRAPPC13 (46)	NP_001087224.1	III

Table 1.1 Nomenclature of mammalian TRAPP subunits indicating the complexes in which they are found.

TRAPPC13, which have no homologues in *S. cerevisiae* (Scrivens et al., 2011; Bassik et al., 2013). TRAPPC2 has been shown to be the main adaptor to mediate the interaction between both TRAPPC9 and TRAPPC8 (Zong et al., 2012). The fact that TRAPPC9 co-purifies with TRAPPC10 but not TRAPPC8 indicates the existence of at least two distinct mammalian TRAPP complexes (Zong et al., 2011). This idea was reinforced by another study where, based on ricin susceptibility and immunoprecipitation studies, it was found that there are two physically distinct complexes: TRAPPII that contains the core subunits (C1-C6) plus TRAPPC9 and 10, and TRAPPIII that contains the core plus TRAPPC8, 11, 12 and 13 (Table 1.1) (Bassik et al., 2013). Furthermore, TRAPPIII as a whole has been suggested to play an important role in human papillomavirus (HPV) infection since depletion of TRAPPC8, 11 and 12 was shown to facilitate the escape of the HPV genome from the Golgi compartment while the depletion of TRAPPC9, a component of mammalian TRAPPII, showed little or no effect (Ishii et al., 2013). The function of TRAPP as a tethering factor is complemented by its ability to act as a guanine nucleotide exchange factor (GEF) for the small GTPase Ypt1/Rab1. All three forms of TRAPP have been shown to be GEFs for Ypt1p in yeast and TRAPPII may be a GEF for Ypt31p/Ypt32p (Jones et al., 2000; Wang et al., 2000; Chen et al., 2011; Yamasaki et al., 2009; Lynch-Day et al., 2010). Mammalian TRAPP was shown to have GEF activity towards the Ypt1p homologue Rab1 (a small GTPase that regulates ER-to-Golgi and early Golgi trafficking) but not the Ypt31p/Ypt32p homologue Rab11 (Yamasaki et al., 2009). ER-to-Golgi trafficking in mammalian cells differs from yeast due to the fusion of COPII vesicles derived from the ER forming the ER-Golgi intermediate compartment (ERGIC) (Appenzeller-Herzog and Hauri, 2006). Importantly, components of mammalian TRAPP have been localized to the ERGIC and have been implicated in ER-Golgi transport (Scrivens et al., 2011; Yu et al., 2006; Zong et al., 2012). In addition, depletion of other TRAPP subunits such as TRAPPC11 and

TRAPPC12 have been shown to impair both the Golgi and ERGIC, consistent with the accepted role of TRAPP in ER-to-Golgi traffic (Choi et al., 2011; Scrivens et al., 2009, 2011).

Like the yeast TRAPP III complex, mammalian TRAPP III has also been implicated in autophagy. A large-scale screen for genes involved in human autophagy identified TRAPPC5, 8, 11 and 12 as components of the autophagy machinery (Behrends et al., 2010). The research in mammalian TRAPP is still in its infancy but steadily progressing, and its importance for better understanding is shown by the different roles and functions that the complex as a whole or its individual subunits undertake. It is noteworthy that mutations in different TRAPP subunits have been linked to a number of diseases (reviewed in Brunet and Sacher, 2014).

1.7 *Project: The role of phosphorylated TRAPPC12 during mitosis*

The common method to investigate the role of proteins in the cell is usually done by the use of small interference RNA (siRNA). Most of the time knockdowns show a particular phenotype, indicating a role of the depleted protein in the cell. Our laboratory has been studying the mechanism of membrane trafficking with a particular focus on the TRAPP complex. We use siRNA knockdowns extensively to examine the phenotypes of individual TRAPP subunits. A general screening of the phenotype resulting from the depletion of different TRAPP subunits by siRNA revealed an unexpected result: depletion of the TRAPPC12 subunit arrested cells in mitosis and this phenotype was not seen for any other TRAPP subunit depletion.

The unexpected finding of the involvement of TRAPPC12 in mitosis led to several questions that I will try to answer in this study. During mitosis many proteins are

exposed to posttranslational modification such as ubiquitination or phosphorylation. In this study, initially I will establish if TRAPPC12 is subjected to posttranslational modifications in order to switch its role from membrane trafficking to mitosis, and then determine the timing of this modification. Afterwards, I will investigate which residues of TRAPPC12 are responsible for its involvement in mitosis and which one of them has the more adverse effect when mutated.

Until now we know that TRAPPC12 is a subunit of TRAPP III complex and is likely to be involved in anterograde traffic between the ER and the Golgi (Scrivens et al., 2011) and possibly in autophagy (Behrends et al., 2010). My present study and recent data from our laboratory suggest it also moonlights as a mitotic protein.

2. Materials and Methods

2.1. Buffers and solutions

All buffers and solutions used in this study are listed in Table 2.1 at the end of this Materials and Methods section.

2.2 Molecular biology techniques

All oligonucleotides, plasmids and DNA constructs generated in this study are listed in Table 2.2 at the end of this Materials and Methods section.

2.2.1 Preparation of DNA and measuring DNA concentration

Plasmid DNA was extracted using a Bio Basic miniprep kit following the instructions supplied by the manufacturer. To measure the concentration, DNA was diluted 100 fold in dH_2O and 500 µl was added to a quartz micro-cuvette. The absorbance was measured using an Ultrospec 2100pro Spectrophotometer at wavelengths of 260 and 280 nm.

2.2.2 Site Directed Mutagenesis with Stratagene *Pfu* Turbo

Site-specific mutagenesis was used to create most of the TRAPPC12 mutants. The plasmid pDONOR-TRAPPC12siRNA-resistant was used as a template and the oligonucleotides are listed in Table 2.2. Site-specific mutagenesis was carried out using the QuikChange® XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol with some changes listed below. Briefly, a typical mutagenesis reaction contained 33.5 µl ddH_2O, 5 µl 10X Pfu Turbo Reaction mix, 2 µl 2.5 mM dNTP, 2.5 µl DMSO, 2 µl Primer1 mix (final concentration of 0.4 µM), 2 µl Primer2 mix (final 0.4 µM), 2 µl Template DNA (~200 ng), 1 µl Pfu turbo DNA Polymerase. The mixture was

subjected to the following protocol in a thermal cycler: 19 cycles of denaturation (95°C for 20 seconds), annealing (55°C for 30 seconds), elongation (68°C for 10 minutes), preceeded by an initial denaturation for 2 minutes. After this protocol, the sample was incubated with 1µl of DpnI overnight at 37°C. 5 µl of the DpnI-digested mixture was used for transformation into subcloning efficiency DH5α competent cells.

2.2.3 Transformation of Bacteria

Transformation of DNA constructs into DH5α competent cells (Invitrogen) or DM1 cells (Invitrogen) was performed as follows. Cells were thawed on ice and 80 µl aliquots were mixed with the appropriate amount of DNA (either a recombination reaction or a ligation reaction) by gentle pipetting. Samples were incubated on ice for 30 minutes, heat shocked at 42°C for 1 minute and placed on ice for 5 minutes. After addition of 1 mL of LB, the transformation mixture was incubated at 37°C for 1 hour at 220 rpm in a shaking incubator. Cells were pelleted at 2500 rpm for 2 minutes then resuspended in 200 µl of LB broth and spread onto LB-agar plates containing ampicillin or kanamycin. Plates were incubated overnight at 37°C and analyzed the next morning.

2.2.4 Plasmid preparation by Gateway® Cloning

Gateway® cloning was used to insert the amplified gene of interest into the entry vector pDONR201 by a BP recombination reaction, and then transferred into the destination vector containing a V5 or FLAG-tag by an LR recombination reaction. All oligonucleotides used in this study are listed in Table 2.2. Briefly, the gene of interest was amplified by polymerase chain reaction (PCR) with oligonucleotides (Table 2.2) containing the attB1 and attB2 recombination sequences. A 3.5 µl BP reaction, containing 45 ng entry vector pDONR201, 45 ng PCR product, 0.5 µl BP Clonase

Enzyme and TE pH 8.0, was set up and incubated at 25°C overnight. The entire reaction mix was transformed into 80 µl of subcloning efficiency DH5α competent cells as described above. The transformation was plated onto LB plates containing 100 µg/ml kanamycin. Once a successful insertion into the entry vector was verified by restriction enzyme digestion and DNA sequence analysis, an LR recombination reaction was performed to transfer the gene of interest from the entry vector to a pcDNA3.1-based destination vector (untagged, FLAG-, V5- or YFP-tagged). Briefly a 3.5 µl reaction, containing 45 ng destination vector, 45 ng entry clone, 0.5 µl LR Clonase Enzyme and TE pH 8.0 to was set up and incubated at 25°C overnight. The entire reaction was transformed into 80 µl of subcloning efficiency DH5α competent cells as above. The transformation was plated on LB plates containing 100 µg/ml ampicillin. The Amp-resistant colonies were digested with the restriction enzyme BsrGI to verify successful recombination.

2.2.5 Restriction enzyme digestion

In some cases, restriction enzyme cloning was used. All restriction enzymes and their respective 10X buffers were obtained from New England Biolabs, USA. Restriction enzyme digestion was used to prepare or check DNA inserts for subcloning. To prepare DNA inserts, plasmid DNA was mixed with restriction enzyme, 10X buffer, and dH_2O. All the reactions were incubated at 37°C for 1 hour. Bovine serum albumin (BSA) (1 µl of 10 mg/ml) was added to some restriction enzyme reactions as instructed by the manufacturer.

2.2.6 Agarose gel Electrophoresis

Analysis of DNA was performed on agarose gels prepared with 70ml of TBE with 1% w/v agarose and ethidium bromide (final concentration of 0.5 µg/ml). 6X DNA loading solution was added to the samples at a final concentration of 1X prior to loading in the gel. Gel electrophoresis was performed in 1X TBE at 100V. DNA was visualized by exposure to UV light.

2.2.7 Gel extraction of DNA

In order to purify DNA either generated by PCR or following digestion with restriction enzymes, samples were first resolved by agarose gel electrophoresis. The DNA of interest was excised and then purified using Bio Basic Gel extraction kit as per the manufacture's instructions.

2.2.8 Ligation

The T4 DNA ligase (400 units/µl) and 10X buffer used in all ligation reactions was obtained from New England Biolabs, USA. Insert and vector were mixed at a 3:1 molar ratio, together with 10X buffer, 200 Units T4 DNA ligase and dH_2O. The mixture was then incubated at room temperature overnight before transformation into competent bacteria.

2.3 Tissue culture techniques

A list of all small interfering RNAs (siRNA) and plasmids used in this study are listed in Tables 2.3 and 2.2 respectively at the end of this Materials and Methods section.

2.3.1 Tissue culture media and solutions

All tissue culture reagents were presterilised, stored at 4°C and pre-warmed to 37°C unless otherwise specified. Cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine. Before use, all media were supplemented with 10% fetal bovine serum (FBS) (Wisent) unless otherwise stated.

2.3.2 Maintenance of cell cultures

Cells were grown in a humidified incubator with 5% carbon dioxide at 37°C. Cell lines were maintained in DMEM. To subculture cells, medium was removed when cells reached confluence, and the cells were washed twice in PBS. 3 ml of 0.05 % Trypsin solution was added to the cells, which were then incubated at 37°C for 2-3 minutes. The trypsin was carefully removed without disturbing adherent cells, after which cells were detached with media containing FBS. The re-suspended cells in DMEM were re-plated to the required density.

2.3.3 Cryopreservation of cells

Cells were trypsinised and pelleted by centrifugation at 1900 rpm for 2 minutes. Each confluent plate was divided into 4-5 aliquots and each aliquot re-suspended in 1ml of cold DMEM-FBS with 10% v/v dimethyl-sulphoxide (DMSO). The cells were stored in sterile ampoules, and placed in freezing boxes at −80°C over night, allowing cooling at a controlled rate of ~1°C/minute. The ampoules were then transferred to a liquid nitrogen storage tank. To recover cells from liquid nitrogen, cell suspensions were thawed quickly in a 37°C water-bath followed by immediate drop-wise transfer to a plate containing 10ml of DMEM-FBS media. The cells were then incubated at 37°C.

2.3.4 Cell synchronization

Various techniques were performed to synchronize HeLa cells in different stages of the cell cycle:

Double thymidine block to arrest cells at the G1/S boundary: cells were treated with 2 mM thymidine (Sigma) for 18 hours, washed 2 times in PBS and released in DMEM for 6 hours, treated a second time with 2 mM thymidine for 16 hours, washed 2 times in PBS and released in DMEM for the times indicated in the Results section.

Colcemid treatment to arrest cells in prometaphase: cells were treated with 5 µg/mL Colcemid (Gibco KaryoMAX) for 18 hours, collected with shake off, washed 2 times in PBS and either released into DMEM or immediately harvested.

Nocodazole treatment to arrest cells in prometaphase: cells were treated with 100 ng/ml Nocodazole (Sigma) for 18 hours, washed 2 times in PBS and released into DMEM, or in some cases, after a double thymidine treatment cells were released into DMEM media for 3 hours and then 100 ng/ml Nocodazol was added before harvesting at different time points. Cell lysates were then analyzed by Western blotting.

2.3.5 DNA and siRNA transfection of mammalian cells using JetPRIME

At the time of transfection cells were 60% confluent. The transfection was performed using JetPRIME (Polyplus) as per the manufacture's instructions. Briefly, for a single well of a 6-well plate, 1µg DNA or 10 nM siRNA was diluted into 200 µl of JetPRIME buffer followed by the addition of 4 µl JetPRIME reagent. After vortexing for 10 seconds and a brief centrifugation in a microcentrifuge the transfection mix was incubated for 20 min at RT. The transfection mix was then added drop-wise to the cells in regular cell growth medium and the cells were returned to the incubator. The medium was replaced 24 hours after transfection.

2.4. Protein chemistry techniques

A list of all antibodies used in this study is listed in Tables 2.4 at the end of this Materials and Methods section.

2.4.1 Protein concentration by Bradford assay

A small volume (1-10 µl) of the protein sample was carefully diluted and mixed with 1 ml of Bradford reagent (BioRad). The absorbance was measured in an Ultrospec 2100pro spectrophotometer at 595 nm against 1ml of Bradford reagent blank. Protein concentrations were determined by comparison with a standard calibration curve prepared from known quantities of BSA (0-10 µg).

2.4.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

For analysis by Western blotting, cells were harvested with lysis buffer then diluted with 4X SDS sample buffer. 5 – 40 µg of whole cell lysates were electrophoresed on 8% (>50kDa) or 15% (< 50kDa) polyacrylamide gels. The samples were typically electrophoresed at 120V in running buffer. Proteins resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to nitrocellulose membranes using standard procedures (Sambrook & Russell, 2001) at 30 V overnight. After blocking with 5 % (w/v) skim milk powder or 5 % BSA (bovine serum albumin), membranes were incubated for 2 hours with primary antibodies (Table 2.4) in PBSt. After 2 washes for 5 minutes each, membranes were incubated for 1 h at room temperature with peroxidase-labeled goat anti-mouse/rabbit IgG (Kirkegaard & Perry Laboratory) secondary antibody at a dilution 1:10000 and then the antigen-antibody complex was visualized using the enhanced chemiluminescence (ECL) detection reagent (Amersham Life Science, Buckinghamshire, England), following the protocols provided by the manufacturer.

2.4.3 Lambda phosphatase treatment

Cells were lysed in lysis buffer and centrifuged at 16,000 rpm for 15 minutes at 4°C. Total cell extracts were incubated with Lambda phosphatase solution (New England Biolabs, Inc.) containing 400 units of Lambda phosphatase (λPPase), 1x λPPase buffer and 2 mM $MnCl_2$. The reaction mixture was incubated for 2 hours at 30°C. The reaction was terminated by the addition of 4 X SDS-sample buffer. The samples were boiled at 100°C for 2 minutes before SDS-PAGE and western blotting analysis.

2.4.4 Gel Filtration chromatography

For gel filtration experiments, HeLa cells were lysed with Lysis buffer. Using the GE ÄKTA FPLC chromatography system, 5 mg of total cell extract was loaded onto a Superose 6 preparation grade column (GE Healthcare) at a flow rate of 0.5 ml/min. The column was pre-equilibrated with 2 volumes of gel filtration buffer. Fractions of 0.5 ml were collected and resolved by SDS-PAGE and western blotting analysis.

2.5 Microscopy

Images were captured with a Nikon Eclipse TS1000 Epi-Fluorescence microscope using a 20X objective lenses (ELWD 20X/0.45), and then ImageJ was used for cell counting.

Table 2.1. Buffers and solutions used in this study

Reagent name	Components
Ampicillin	100 mg/mL in dH2O
dNTP solution (10x)	2 mM each of dATP, dCTP, dGTP and dTTP
DNA loading solution 6X	30%v/v of glycerol, 0.25% w/v of bromophenol blue in dH2O
Gel filtration buffer	50mMTris pH7.2, 150mM NaCl, 0.5mM EDTA, 1mM DTT
Kanamycin	100 mg/mL in dH2O
Luria Broth (LB)	1% w/v tryptone, 0.5% w/v yeast extracts, 1% w/v NaCl
LB-agar plates	LB with 1.5% agar
Mammalian lysis buffer	50mMTris pH7.2, 150mM NaCl, 0.5mM EDTA, 1mM DTT, 1% Triton X-100(v/v), 1 tablet of protease inhibitor cocktail (Roche) and 2 tablets of Phospho-Stop (Roche) per 10mL.
PBS	0.8% w/v NaCl, 0.02% w/v KCl, 0.061% w/v Na_2HPO_4, 0.02% w/v KH_2PO_4, pH 7.3
PBSt	PBS with 0.1% Tween-20 (v/v)
SDS-PAGE running buffer	25mM Tris-base, 200mM glycine, 0.1% SDS
4XSDS sample buffer	80 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% v/v glycerol, 0.1% bromophenol blue, 5% v/v β-mercaptoethanol
TBE	100mM Tris, 100mM boric Acid, 2mM EDTA, pH 8.3
TE	10 mM Tris, pH 8.0 , 1 mM EDTA
Western blotting transfer buffer	25mM Tris-base, 200mM glycine, 20% methanol

Table 2.2 List of Oligonucleotides and the constructs that were generated in this study.

OLIGOS	SEQUENCES	Plasmid
C12R-GWYN-term	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAGATGTGCGCGAAAGGCTCTGG	C12 WT
C12F-GWYC-term	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCCAGAGCCTTTCGCGCACATC	
pDONR201-F-seq	TCGCGTTAACGCTAGCATGGATCTC	C12 Nterm
C12R-GWYN-term	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAGATGTGCGCGAAAGGCTCTGG	
pDONR201-R-seq	GTAACATCAGAGATTTTGAGACAC	C12 Cterm
C12F-GWYC-term	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCCAGAGCCTTTCGCGCACATC	
C12T107A-F	GCCCGGAGCCCGCGGGCGCCCCGAGTCCCAGCGGCGAGGCCGACGG	C12 T107A
C12T107A-R	CCGTCGGCCTCGCCGCTGGGACTCGGGGCGCCCGCGGGCTCCGGGCC	
C12S109A-F	GGCCCGGAGCCCGCGGGCACCCCGGCTCCCAGCGGCGAGGCCGACGG	C12 S109A
C12S109A-R	CCGTCGGCCTCGCCGCTGGGAGCCGGGGTGCCCGCGGGCTCCGGGCC	
C12S127A-F	GAGGACGCGGCACCCAGTGCCGGAGGGGCCCCGAGGCAGG	C12 S127A
C12S127A-R	CCTGCCTCGGGGCCCCTCCGGCACTGGGTGCCGCGTCCTC	
C12S182A-F	CGAGCCGCAGATGGTGAAGGCGCCCAGCTTCGGTGGCGCCAGCG	C12 S182A
C12S182A-R	GCTGGCGCCACCGAAGCTGGGCGCCTTCACCATCTGCGGCTCG	
C12S184A-F	GAGCCGCAGATGGTGAAGTCGCCCGCCTTCGGTGGCGCCAGCG	C12 184A
C12S184A-R	CGCTGGCGCCACCGAAGGCGGGCGACTTCACCATCTGCGGCTCG	
107/109-DD-F	CGGAGCCCGCGGGCGACCCGGATCCCAGCGGCGAGGC	C12 TS107/109DD
107/109-DD-R	GCCTCGCCGCTGGGATCCGGGTCGCCCGCGGGCTCCG	
109-D-F	CGGAGCCCGCGGGCACCCCGGATCCCAGCGGCGAGGC	C12 S109D
109-D-R	GCCTCGCCGCTGGGATCCGGGGTGCCCGCGGGCTCCG	
107-D-F	CGGAGCCCGCGGGCGACCCGAGTCCCAGCGGCGAGGC	C12 T107D
107-D-R	GCCTCGCCGCTGGGACTCGGGTCGCCCGCGGGCTCCG	
C12-TS107/109AA-F	CGGAGCCCGCGGGCGCCCCGGCTCCCAGCGGCGAGGC	C12 TS107/109AA
C12-TS107/109AA-R	GCCTCGCCGCTGGGAGCCGGGGCGCCCGCGGGCTCCG	
C12 S182/184D-F	CGAGCCGCAGATGGTGAAGGATCCCGACTTCGGTGGCGCCAGCG	C12 SS182/184DD
C12 S182/184D-R	CGCTGGCGCCACCGAAGTCGGGATCCTTCACCATCTGCGGCTCG	
C12-TPR-R-EcoRI	CCGGAATTCCTACAGACGTGACCTCCACAGCCGGATAG	C12 NoTPR
C12-F-HindIII	CCCAAGCTTACCATGGACTACAAGGATGACGATGACAAAATGGAGGACGCTGGCGGCGGCGAGG	

Table 2.3 List of siRNA's used in this study

Targeting gene	Code	Sequence (5' to 3')
Mad2L1HSS106245	7643164 (Invitrogen)	GGGAAGAGUCGGGACCACAGUUUAU
TRAPPC2	s12673 (Ambion)	CAAUUCUCCUAUUCGAUCAtt
TRAPPC2L	s28534(Ambion)	AGCCCUUCGAGACAACGAAtt
TRAPPC4	s28090(Ambion)	CGAAAGAUUUAUGAGAUUUtt
TRAPPC8	s22569(Ambion)	CAGCUCUCCUAAUACGGUUtt
TRAPPC9	s38117(Ambion)	GGAGAAAGUCAGCAACUAAtt
TRAPPC10	(Cedar Lane)	GUGCCAACUGGCUgacttttt
TRAPPC11	s226950 (Ambion)	GGAUUUAUAAACUACAAGAtt
TTC15	s27465 (Ambion)	CGGACAAGCUGAACGAACAtt
TRAPPC13	s228222(Ambion)	GCAAUCAAGUUGUAAAAGAtt

Table 2.4. List of antibodies used in this study

Antigen	Host	Code	Source	Dilution
Cyclin B1	Rabbit	4138P	Cell Signaling Technology	1:1000
Flag	Mouse	M2	Sigma	1:10000
Histone H3 (phosphor-S10)	Rabbit	3377P	Signaling Technology	1:1000
TTC15	Mouse	Ab88751	Abcam	1:1000
Tubulin	Mouse	Ab27076	Abcam	1:5000
V5	Mouse	Ab27671	Abcam	1:5000

3. RESULTS

3.1. Depletion of TRAPPC12 leads to a mitotic arrest

In order to investigate the role of each subunit of the mammalian TRAPP complex in membrane trafficking, small interfering RNA (siRNA) knockdown was used to deplete HeLa cells of individual proteins. Depletion of the TRAPPC12 subunit, but no other tested TRAPP subunit, showed an increase in the number of round cells that is a hallmark of a cell in mitosis (Figure 3.1). Upon closer investigation by brightfield microscopy it is clear that the majority of the TRAPPC12-depleted cells were arrested in metaphase with a noticeable formation of a metaphase plate (not shown). This phenotype has a striking similarity with the depletion of CENP-E from cells as seen in previous studies (McEwen et al., 2001; Yao et al., 2000). Depletion of CENP-E results in a failure in metaphase chromosome alignment due to defects in kinetochore-microtubule attachment (Mcewen et al., 2001; Schaar et al., 1997; Yao et al., 2000). Quantification of different fields from different knockdown experiments revealed an increase of the mean mitotic index for the TRAPPC12-depleated cells (29.7%±2.3) compared to cells treated with a non-specific RNA (6.4%±0.43) (Figure 3.1B). This unexpected finding suggests that TRAPPC12 may be involved in mitosis.

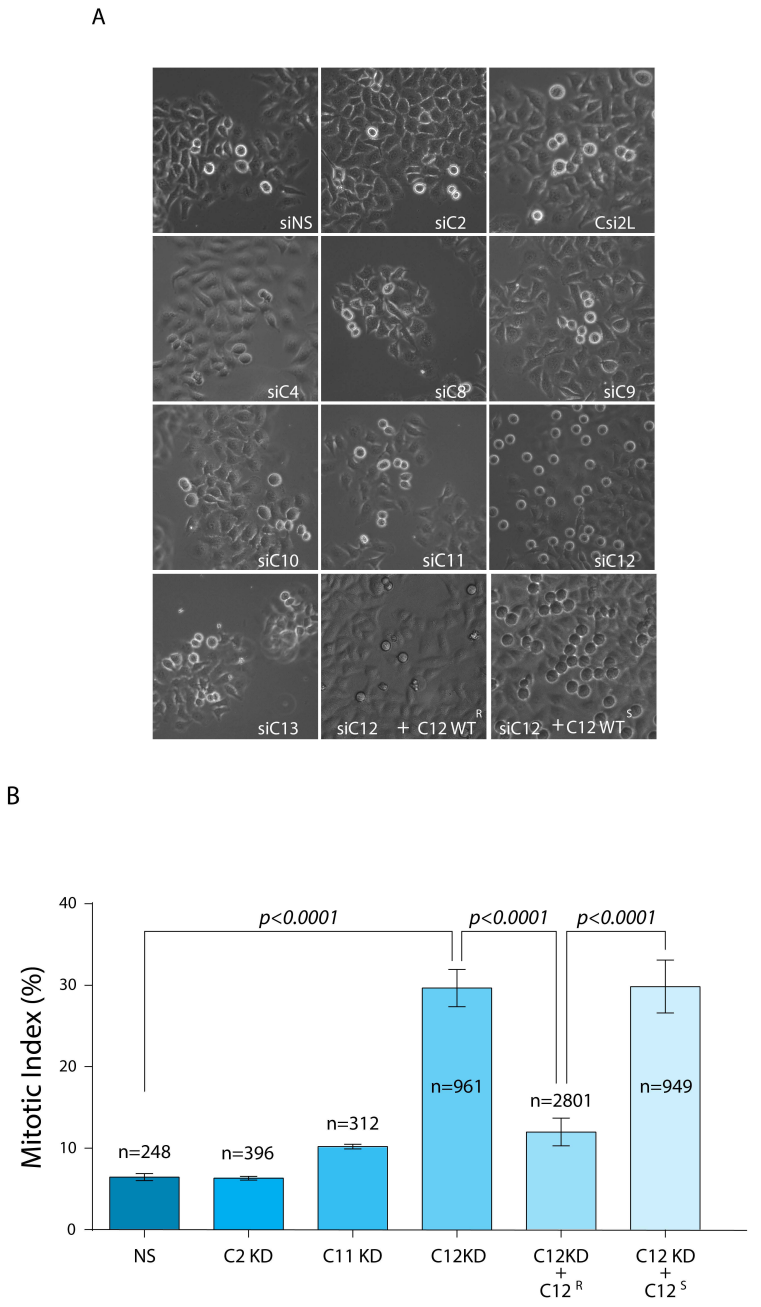

Figure 3.1. Depletion of TRAPPC12 results in a mitotic arrest. A) HeLa cells were photographed by brightfield microscopy at 20X magnification 24 hours after treatment with siRNA against the TRAPP subunits indicated. **B**) Cells were quantitated by counting the number of mitotic cells in multiple fields over three independed experiments. Error bars indicate standard deviation. Note that for simplicity “Trapp” is omitted for each subunit, si – small interference RNA, NS – non-specific siRNA.

3.2 Bioinformatics analysis of TRAPPC12

Solving the structure of a protein is an important step toward determining its function. In the absence of a crystal structure, bioinformatic analysis can provide limited information on the protein of interest. BLAST search of TRAPPC12 (NP_057114.5) shows a conserved region of tetratricopeptide repeat (TPR) motifs. TPR motifs are important for protein-protein interactions and are commonly found in cell cycle proteins. These motifs are very diverse with a degenerate sequence of ~34 amino acids but their structure remains conserved. Due to their structure, TPR domains act as ligand binding domains and the proteins that contain them usually support multiprotein complex formation (Scheufler et al. 2000).

For further exploration of this conserved region of TRAPPC12, KEGG database was used. The database resource KEGG (Kyoto Encyclopedia of Genes and Genomes) contains information on proteins and their conserved motifs. Based upon this database, the sequence of TRAPPC12 aligns to varying degrees with different TPR motifs including a match for the anaphase-promoting complex (APC) domain (Figure 3.2A). Anaphase-promoting complex (APC) is an E3 ligase that is involved in mitosis by mediating the degradation of securin and cyclin B1 (Glotzer et al., 1991). It should be noted that the Expect (E) -value for the APC domain is very high, suggesting that the alignment may be by chance (Table 3.1). KEGG results show the presence of 16 potential TPR motifs in TRAPPC12. Since some of them have a high E-value, and APC also contains TPR motifs, this further suggests that the alignment with APC3 maybe by chance (Table 3.1). However, given the role of TRAPPC12 in mitosis this alignment is of potential interest. Based on both KEGG analysis and analysis of the TPR prediction

software TPRpred that are used to detect TPR domains and compute the statistical significance of their occurrence, TRAPPC12 likely contains 4 TPR motifs.

In order to analyze the conservation of the TPR motifs in TRAPPC12 among species, the Treefam database was used to generate a phylogenetic tree. The resulting phylogenic tree shows that the TPR domains are conserved in this protein throughout different species (Figure 3.2B). However, differences have occurred in the evolutionary line demonstrated by the absence of TPR motifs in C.*elegans* (Figure 3.2B). The absence of a TPR domain in *C. elegans* and other worms suggests that the repeat may have been expanded independently in these organisms or that the TPR motif was not detected by the program due to diverse and degenerate nature of these motifs.

The Phyre 2 database was then used to predict the tertiary structure of TRAPPC12. The result showed that TRAPPC12 is composed of 44% alpha helices, mostly at the C-terminus, and 53% is unstructured, mostly at the N-terminus. Based on the resolved structures of the similar domains that match the TPR and other known domains at the C-terminus of the protein, the program was able to elucidate a structure for TRAPPC12 (Figure 3.2C). The predicted structure of TRAPPC12 shows that the TPR domain (Figure 3.2C, blue) is exposed on the outside of the protein suggesting an easy way to facilitate protein-protein interaction. Furthermore, the presence of the unstructured region at the N-terminus (Figure 3.2C, pink) is noteworthy for several reasons. First, unstructured regions have been shown to facilitate bindings that are controlled by post translational modification. This is commonly seen in proteins involved in cell signalling and chromatin remodelling (Collins et al., 2008; Iakoucheva et al., 2002; Sandhu, 2009). Second, as will be described in detail, it is this region of TRAPPC12 that is phosphorylated during mitosis (see section 3.3). Overall, the results of the

bioinformatic analysis can help in the prediction of key areas that through mutations can reveal some function of TRAPPC12.

Motif id	From	To	Definition	E-value
TPR_1	547	577	Tetratricopeptide repeat	0.0016
TPR_2	547	577	Tetratricopeptide repeat	0.14
TPR_2	623	653	Tetratricopeptide repeat	0.0065
TPR_2	656	687	Tetratricopeptide repeat	0.21
TPR_6	547	577		0.0058
TPR_11	549	604		9.90E-05
TPR_11	626	685		4.40E-08
TPR_12	545	609		0.0011
TPR_12	581	650		0.015
TPR_12	622	687		0.23
TPR_14	627	661		0.75
TPR_16	555	605		0.23
TPR_16	627	686		2.50E-05
TPR_17	643	673		0.094
TPR_19	555	612		0.3
TPR_19	631	690		3.90E-06
Apc_3	547	604	APC, subunit 3	0.096
Apc_3	595	678	APC, subunit 3	0.33
Coatomer_E	617	691	Coatomer epsilon subunit	0.00061

Table 3.1. TPR Motifs extracted from the KEGG analysis found in TRAPPC12.

http://www.kegg.jp/ssdb-bin/ssdb_motif?kid=hsa:51112

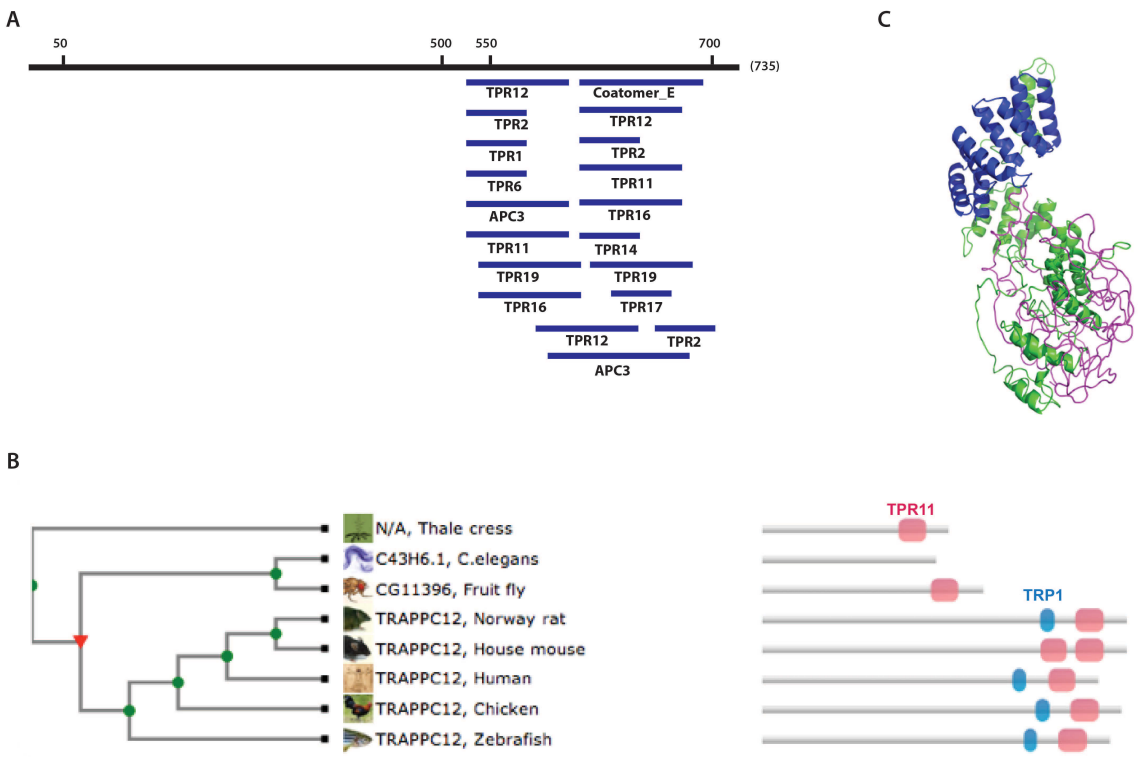

Figure 3.2. Structural analysis of TRAPPC12. **A)** TPR Motifs extracted from the KEGG analysis found in TRAPPC12. The database resource KEGG (Kyoto Encyclopedia of Genes and Genomes) of TRAPPC12 (ID number hsa: 51112) shows different TPR domains and the presence of the anaphase-promoting complex APC3 domain. http://www.kegg.jp/ssdb-bin/ssdb_motif?kid=hsa:51112

B) The TreeFam family. The sequences were aligned using MCoffee. TreeBest (Ruan *et al* 2008) was used to build a gene tree and reconcile it with the species tree. TreeBest built 5 source trees (Guindon *et al* 2010), and then merged them into one tree trying to minimize the number of duplications using a species tree. Legend: pink and blue rectangles signify the TPR domains in the sequences. http://www.treefam.org

C) Tertiary structure of TRAPPC12 predicted by the Phyre2 program. The amino-terminus is coloured in pink and the TPR repeat domain in blue.

http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index

3.3. TRAPPC12 is hyperphosphorylated during mitosis

The increase in mean mitotic index (see section 3.1) from the siRNA knockdown experiment led to further investigation of the role of TRAPPC12 during mitosis. Western blot analysis of the cells arrested in prometaphase with the use of a drug called colcemid showed that TRAPPC12 migration on a polyacrylamide gel was retarded (Figure 3.3A). The colcemid-induced arrest in mitosis is indicated by the appearance of the phosphorylated histone H3 that is present only during mitosis (Figure 3.3A) (Hendzel et al., 1997). A reduction in electrophoretic mobility is often an indication of phosphorylation of a protein and many proteins that function during mitosis are subjected to this type of posttranslational modification. To test if this was occurring to TRAPPC12, lysates from asynchronous cells, cells arrested at the G1/S boundary by thymidine treatment and cells arrested in prometaphase using colcemid were treated with lambda protein phosphatase. Lambda protein phosphatase has an activity towards phosphorylated serine, threonine and tyrosine residues. The Western blot analysis showed that the reduced mobility of TRAPPC12 was reversed by lambda phosphatase treatment of colcemid -treated cells indicating that this protein is hyperphosphorylated during mitosis (Figure 3.3B). Interestingly, arrested cells at the G1/S boundary also showed to be dephosphorylated to a lesser extent by lambda phosphatase indicating that TRAPPC12 may be consequently phosphorylated at some sites (Figure 3.3B).

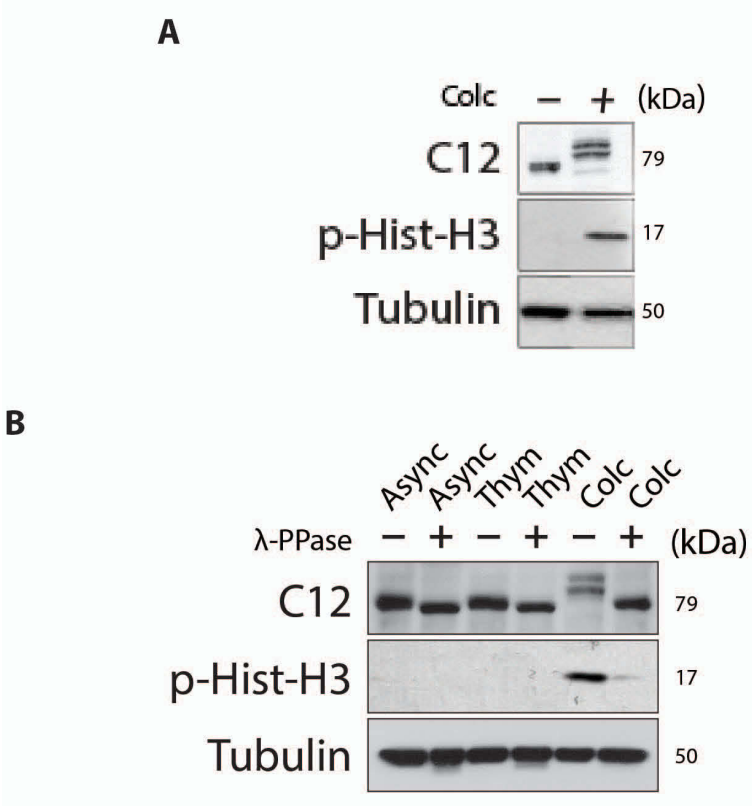

Figure 3.3. TRAPPC12 is hyperphosphorylated during mitosis. A) Lysates were prepared from HeLa cells that were untreated or treated with colcemid to arrest the cells in prometaphase (Colc) and subjected to western analysis. Tubulin was used as a loading control and phospho-histone H3 (p-Hist-H3) was used as a mitotic marker. **B)** Lysates were prepared from HeLa cells that were untreated (Async), treated with thymidine to arrest the cells at the G1/S boundary (Thym), or treated with colcemid to arrest the cells in prometaphase (Colc). A portion of each lysate was left untreated (-) or treated with lambda phosphatase (λ-PPase) (+) prior to western analysis. Tubulin was used as a loading control and phospho-histone H3 (p-Hist-H3) was used as a mitotic marker.

3.4. TRAPPC12 is transiently phosphorylated during mitosis

After establishing that TRAPPC12 is phosphorylated during mitosis, the question addressed was the timing of the mitotic phosphorylation and to determine when dephosphorylation occurs. To answer this, time course experiments were conducted as follows. HeLa cells were treated with thymidine to synchronize the cells at the G1/S boundary and then released into medium containing nocodazole to trap the cells in mitosis. Cell lysates were collected at different time points after treatment with nocodazole and subjected to western blot analysis. At approximately 11 hours after release from the G1/S boundary, TRAPPC12 showed signs of phosphorylation indicated by the appearance of the higher molecular weight form of the protein (Figure 3.4A). Molecular markers like cyclinB1 and phospho-histone H3 were used to determine at what time the phosphorylation of TRAPPC12 occurs. CyclinB1 is present at low concentrations at G1/S phase but its levels start increasing during prophase reaching its peak at metaphase (Jackman et al., 2003). Histone 3 phosphorylation initiates during late G2 and continues until anaphase when its dephosphorylation starts (Hendzel et al., 1997). Based on these molecular markers the phosphorylated form of TRAPPC12 appears as the cells enter mitosis. To determine when the dephosphorylation of TRAPPC12 occurs, HeLa cells were treated with nocodazole for 18 hours to block the cells in mitosis and then mitosis was allowed to progress by releasing the cells into regular medium without nocodazole. Cell lysates were collected at different time points after release and subjected to western blot analysis. The disappearance of the phosphorylated form of TRAPPC12 occurs at approximately 4 hours after release (Figure 3.4B). This coincides with the degradation of cyclinB1, which occurs during anaphase (Figure 3.4B). Collectively, this data suggests that the phosphorylation of TRAPPC12 starts early in mitosis and continues until the onset of anaphase.

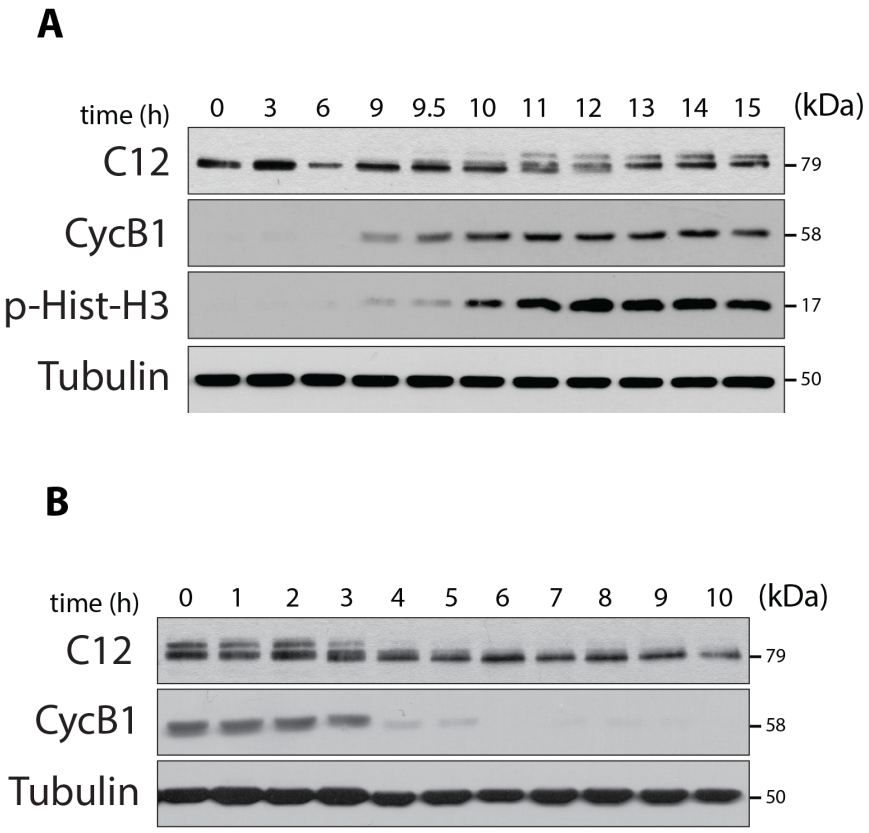

Figure 3.4. Transient phosphorylation of TRAPPC12 during mitosis. A) HeLa cells were arrested at the G1/S boundary by treatment with thymidine. A sample was removed (0 hours) and then the cells were transferred to growth medium containing nocodazole (see Materials and methods) at which point samples were removed at the times indicated (hours). **B)** HeLa cells were arrested in prometaphase by treatment with nocodazole. A sample was removed (0 hours) and then the cells were transferred to regular growth medium at which point samples were removed at the times indicated (hours). The cells were lysed and examined by western analysis for the proteins indicated. Tubulin was used as a loading control and phospho-histone H3 (p-Hist-H3) or CyclinB1 (CycB1) was used as mitotic markers.

3.5. Phosphorylated residues of TRAPPC12 that may be involved in mitosis.

In order to determine which residues in TRAPPC12 are phosphorylated during mitosis, a combination of mass spectrometry, bioinformatics predictions and previously published phosphoproteomic analyses was used (Dephoure et al., 2008; Kettenbach et al., 2011; Mayya et al., 2009). The combined approach led to the examination of 5 potential residues: T107, S109, S127, S182 and S184. Using site directed mutagenesis, 11 mutants were generated that contained one or two of these sites changed to either non-phosphorylatable alanine (A) residues or phosphomimetic aspartic acid (D) residues (Figure 3.5A). The mutants were made siRNA-resistant to prevent degradation when treated with siRNA to TRAPPC12. This allowed for expression of the mutants in cells where the endogenous protein was depleted. In order to detect the exogenous proteins they were tagged with a V5 epitope. The ability of these mutants, compared to wild type TRAPPC12, to rescue the TRAPPC12 depletion-induced increase in the mitotic index was then assessed (Figure 3.5B). All of the mutants containing one or two residues changed to alanine or aspartic acid were able to suppress the mitotic index phenotype of TRAPPC12-depleted cells. Some of these results are quantitated in Figure 3.5C. This led to the conclusion that more than two residues are responsible for the involvement of TRAPPC12 in mitosis.

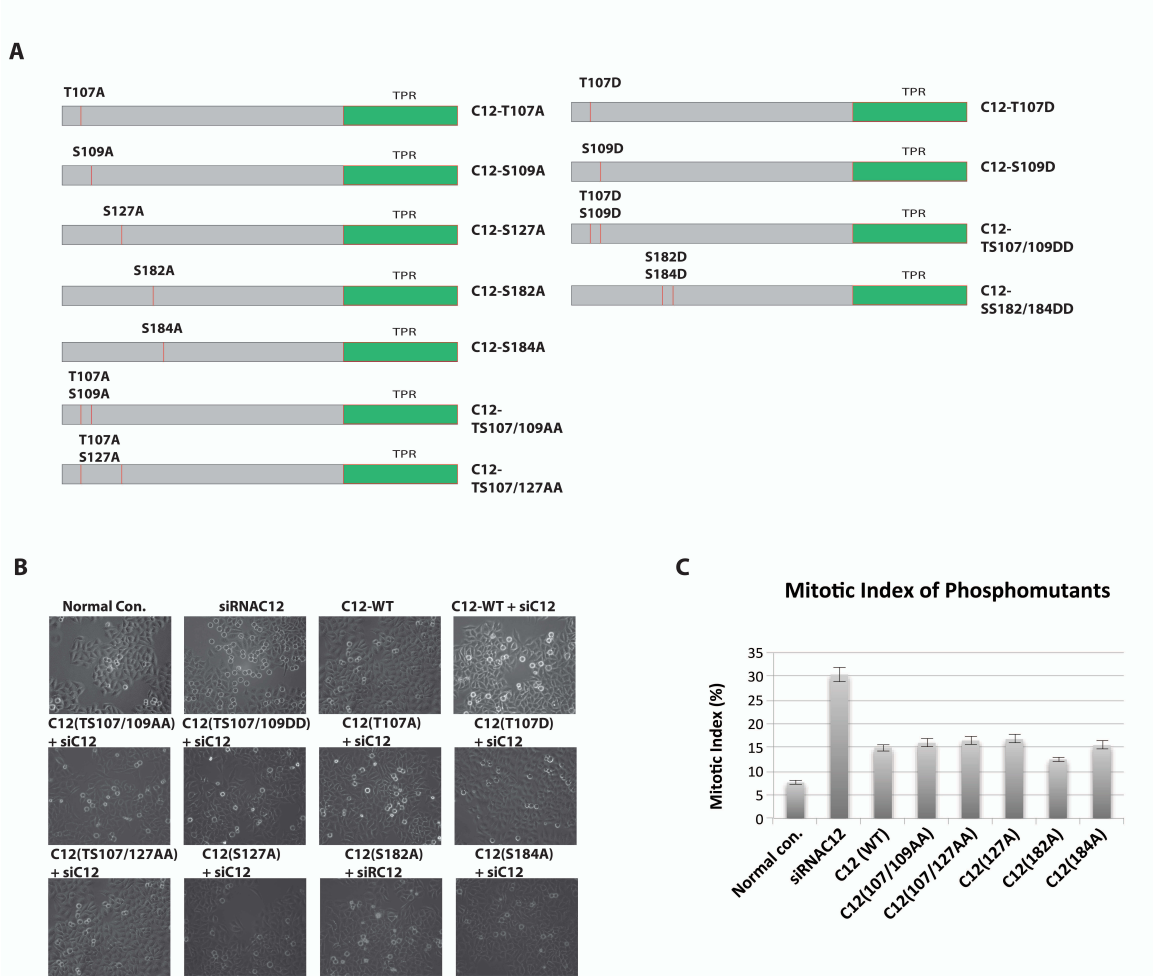

Figure 3.5. Phosphorylated residues of TRAPPC12 that may have a role during mitosis. A) Schematic representation of the different phosphorylation sites of TRAPPC12. The 5 potential residues, T107, S109, S127, S182 and S184 were changed singly or as pairs as indicated in the figure, to either non-phosphorylatable alanine residues (A) or phosphomimetic aspartic acid residues (D) and made siRNA-resistant. **B)** HeLa cells were photographed by brightfield microscopy at 20X magnification 24 hours after transfection with the mutants and/or treatment with siRNA against TRAPPC12 as indicated. **C)** Cells were quantitated by counting the number of mitotic cells in multiple fields over three independent experiments for some of the conditions. si – small interference RNA.

3.6. Reversible phosphorylation of TRAPPC12 is critical for its mitotic function

The results above suggested that more than two residues are involved in the mitotic function of TRAPPC12. Therefore, mutants that contained all five potential residues changed to either non-phosphorylatable alanine (5A) residues or phosphomimetic aspartic acid (5D) residues were constructed (Figure 3.6A). As shown in Figure 3.6B, although wild type TRAPPC12 as well as TRAPPC12-5A could suppress the mitotic index phenotype of a TRAPPC12 depletion nearly equally, TRAPPC12-5D showed a reduced ability to suppress this phenotype. Quantitation of the mitotic index confirmed that the TRAPPC12-depleted phenotype could be suppressed with expression of the wild type and TRAPPC12-5A mutant but only partially by the TRAPPC12-5D mutant (11.9%±0.02 for wild type, 9.8%±0.05 for 5A and 20.0%±0.08 for 5D) (Figure 3.6C). These results suggest that, although phosphorylation of TRAPPC12 is not necessary (based on the TRAPPC12-5A mutant), phosphorylation needs to be reversible for it to function properly (based on the TRAPPC12-5D mutant).

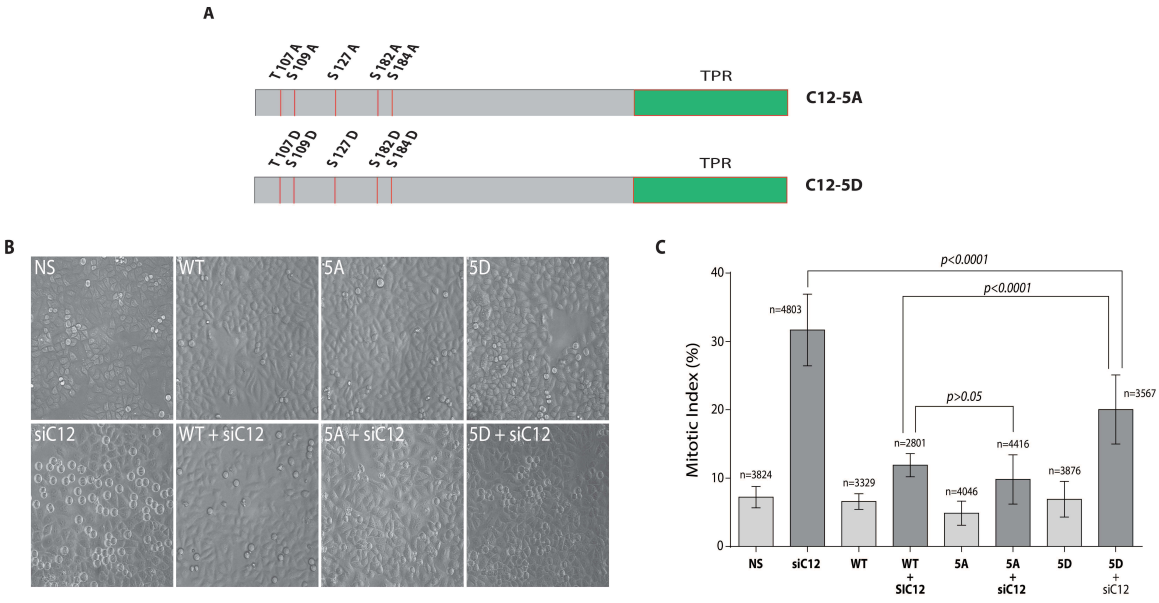

Figure 3.6. Reversible phosphorylation of TRAPPC12 is critical for its mitotic function. A) Schematic representation of two constructs where 5 potential residues (T107, S109, S127, S182 and S184) were changed to either non-phosphorylatable alanine residues (5A) or phosphomimetic aspartic acid residues (5D) and made siRNA-resistant. **B)** HeLa cells were photographed by brightfield microscopy at 20X magnification 24 hours after treatment with siRNA against the TRAPPC12 or non-secific siRNA (NS). In some case, the cells were co-transfected with siRNA-resistant TRAPPC12 WT (wild type), siRNA-resistant TRAPPC12 5A or siRNA-resistant TRAPPC12 5D mutants. Note that for simplicity “Trapp” is omitted. **C**) Cells from panel B were quantitated by counting the number of mitotic cells in multiple fields over three independent experiments. Error bars indicate standard deviation. Significance was assessed by an unpaired t-test.

3.7. Role of TPR domain in the mitotic function of TRAPPC12

After determining that the mitotically phosphorylated sites of TRAPPC12 reside in the unstructured regions of the amino-terminus of the protein (see Section 3.6), the next step was to determine the role of the TPR domain of this protein. TPR motifs are important for protein-protein interactions and are commonly found in cell cycle proteins. TRAPPC12 contains several such domains at the carboxyl-terminus (see Section 3.2). Using site directed mutagenesis, two constructs were established. One of the constructs lacked the TPR domain of TRAPPC12 and was made siRNA resistant since this oligonucleotide targets the amino-terminus of the protein (Figure 3.7, TRAPPC12-NoTPR). The other construct lacked the amino-terminus (Figure 3.7, TRAPPC12-C-term). To test the ability of these two mutants to suppress the TRAPPC12-depleted mitotic index phenotype, HeLa cells were transfected with TRAPPC12-NoTPR and C-term constructs. As seen in Figure 3.7B and C, none of the mutants could suppress the TRAPPC12-depleted phenotype. These results suggest that, the N-terminus of TRAPPC12 is necessary but not sufficient to rescue the mitotic phenotype and the TPR domain may play an important role for this protein either by facilitating interactions with other mitotic proteins or by keeping the structural integrity of the protein.

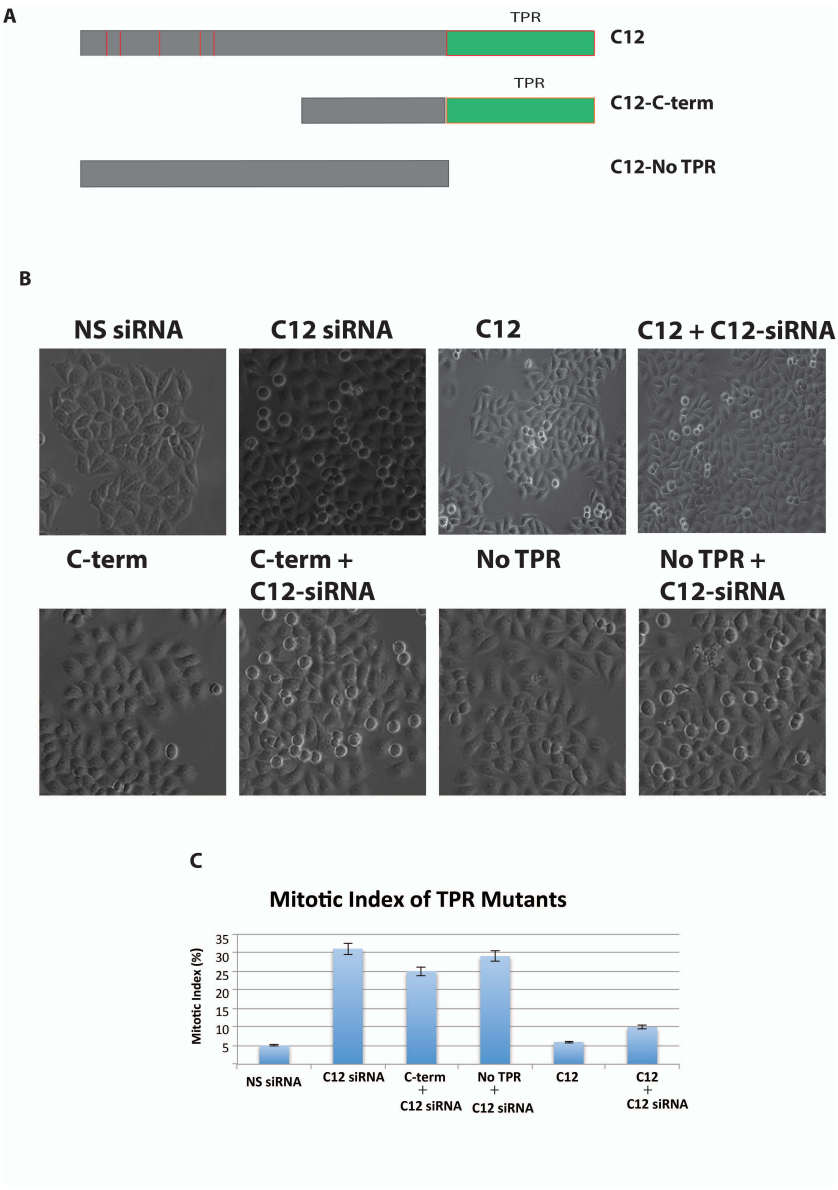

Figure 3.7 Role of the TPR domain in the function of TRAPPC12. A) Schematic representation of two constructs where the TPR domain was removed from the protein (No TPR) or where the TPR domain was expressed alone with a small portion of the protein (C-term). **B)** HeLa cells were photographed by brightfield microscopy at 20X magnification 24 hours after treatment with siRNA against TRAPPC12 or non-specific siRNA (NS). Cells were co-transfected with siRNA-resistant TRAPPC12 NoTPR or TRAPPC12 (C-term). Note that for simplicity "Trapp" is omitted. **C**) Cells from panel B were quantitated by counting the number of mitotic cells in multiple fields over three independent experiments. Error bars indicate standard deviation.

3.8. Size exclusion chromatography of TRAPPC12 mutants

As demonstrated above (Figure 3.1), it is clear that TRAPPC12 is the only component of TRAPP that also functions during mitosis, suggesting that it may be released from the TRAPP complex during this stage of the cell cycle. To examine this, lysates from both asynchronous and colcemid-treated cells were fractionated on a size exclusion column and the molecular size distribution of TRAPPC12 was assessed by western analysis. As seen in Figure 3.8 (top panel) TRAPPC12 from untreated cells displayed a broad size distribution (fractions 19-25) with the larger molecular size fractions corresponding to the TRAPP complex (Bassik et al., 2013). In contrast, TRAPPC12 from colcemid- treated cells displayed a shift to a smaller molecular size (second panel), peaking in fractions 24/25, suggesting that TRAPPC12 separates and is no longer part of the TRAPP complex during mitosis. In addition, the presence of a slower migrating band is visible in untreated cells and this band is only seen in fractions 24/25. These are the same fractions where the mitotic form of TRAPPC12 fractionates, suggesting that during mitosis the hyperphosphorylated form of this protein is not found in the TRAPP complex. HeLa cells were transfected with TRAPPC12-5D or -5A mutants and then treated with colcemid to arrest the cells in prometaphase. Lysates from these cells were fractionated on a size exclusion column and were assed by western blot analysis. The slower migrating band is seen to be present in smaller molecular size of the overexpressed wild type TRAPPC12 and the 5D mutant (third and fifth panel) but not in the 5A mutant (fourth panel) in colcemid-treated cells. Due to overexpression of the protein, no shift in molecular size was detected for the mutants of TRAPPC12 treated with colcemid. Overall, these results suggest that the hyperphosphorylated form of TRAPPC12 shifts at smaller molecular size fractions indicating dissociation from the TRAPP complex during mitosis (as seen in the fractions of WT and 5D mutant).

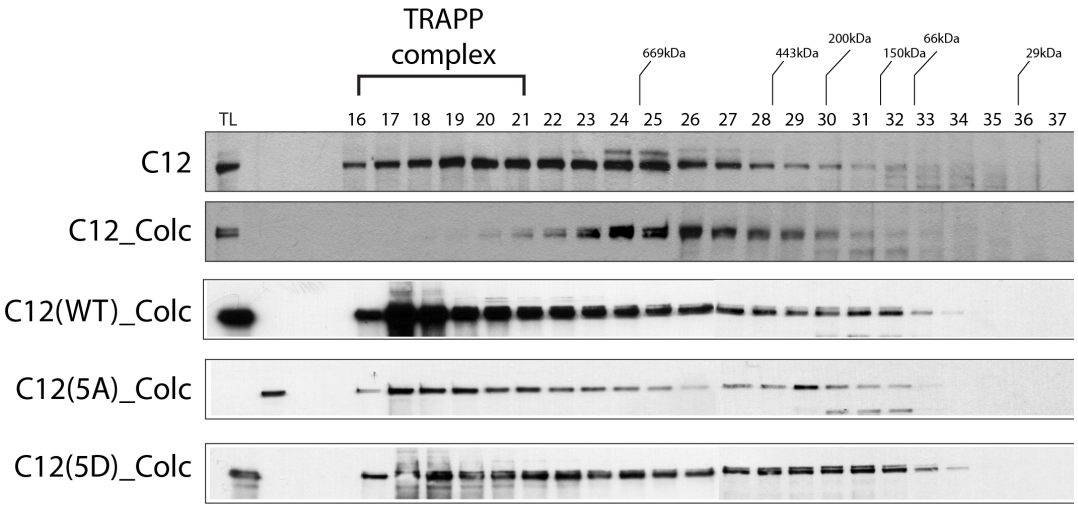

Figure 3.8. Size exclusion chromatography of TRAPPC12-5A and -5D mutants. HeLa cells were left untransfected (top two panels) or transfected with either V5-tagged TRAPPC12 (third panel), TRAPPC12-5A (fourth panel) or TRAPPC12-5D (fifth panel). Some were treated with colcemid (Colc) overnight as indicated. Lysates were fractionated on a Superose 6 size exclusion column and probed for the TRAPPC12 protein with antibodies against TRAPPC12 for the top two panels and with anti-V5 for the rest. The migration of molecular size standards is indicated above the top panel. (colc -cells were treated with colcemid)

4. DISCUSSION

The mammalian TRAPP complex was shown to be involved in various vesicle trafficking pathways outside ER-to-Golgi transport in addition to tethering and guanine nucleotide-exchange (GEF) activity. Indeed, several subunits of mammalian TRAPP have been suggested to have other functions (Brunet and Sacher, 2014). For example, it has been shown that TRAPPC9 mediates the recruitment of essential components during ciliogenesis and the depletion of TRAPPC3, TRAPPC9, or TRAPPC10 damages primary ciliary membrane biogenesis by inhibiting Rabin8 recruitment to the centrosome (Westlake et al., 2011). The ASH (ASPM, SPD-2, Hydin) domains of TRAPPC10 and TRAPPC11 were implicated in microtubule organization and TRAPPC8 was shown to promote ciliogenesis (Schou et al., 2014). TRAPPC4 (also known as synbindin) was found in the spines of neural dendrites where it is thought that, in concert with other proteins, it recruits intracellular vesicles to postsynaptic sites (Ethell et al., 2000).
Work in this study provides evidence to suggest that TRAPPC12, a component of the mammalian TRAPPIII complex, has a role in mitosis. Prior to this study, the only known phenotype of the knockdown of mammalian TRAPP components such as TRAPPC2, TRAPPC2L, TRAPPC8, TRAPPC11, TRAPPC12 and TRAPPC13 was perturbation of Golgi structure (Scrivens et al. 2009; Scrivens et al. 2011; Duarte, 2012 unpublished results).

The observations presented in this study show that depletion of TRAPPC12 arrests cells in a metaphase-like state with a noticeable formation of a metaphase plate, a phenotype not seen for any other TRAPP subunit examined. Depletion of TRAPPC12 shows a considerable increase of the mean mitotic index. This phenotype, as well as other phenotypic features, have a striking similarity with the depletion of CENP-E from

cells (McEwen et al., 2001; Yao et al., 2000). Depletion of CENP-E results in a failure in metaphase chromosome alignment due to defects in kinetochore-microtubule attachment (Mcewen et al., 2001; Schaar et al., 1997; Yao et al., 2000). Hence, this unexpected finding suggests that TRAPPC12 may be involved in mitosis.

TRAPPC12 is a recently characterized protein (Scrivens et al., 2011) that does not yet have a solved crystal structure. Solving the structure of a protein is an important step toward determining its function so in the absence of a crystal structure, bioinformatic approaches were used to provide some information on this protein. A BLAST search showed that TRAPPC12 contains a tetratricopeptide repeat (TPR) domain that is well characterized as a protein-protein interaction domain. Based on both KEGG database and the TPR prediction software TPRpred that are used to detect TPR domains and compute the statistical significance of their occurrence, TRAPPC12 likely contains 4 TPR motifs. Conservation of TRAPPC12 across species was established by building a phylogenic tree of TRAPPC12 protein. The phylogenic tree of TRAPPC12 showed that the TPR domain is conserved in this protein throughout different animal species. However, the C.*elegans* does not contain a TPR motif in TRAPPC12 but the detection may have been missed due to the diversity of the TPR motifs. Also it may be possible that during evolution some eukaryotes with larger genomes tend to have an abundance of domain repeats that possibly aid in more advanced cellular processes. Moreover the absence of a TPR domain in *C. elegans* and other worms suggest that the repeat has been expanded independently in fruit fly and chordates. The predicted structure of TRAPPC12 shows that the TPR domain may be exposed on the outside of the protein suggesting an easy way to facilitate protein-protein interactions. This was further confirmed when the truncated TRAPPC12 protein missing the TPR domain could not function properly, as evidenced by its inability to suppress the TRAPPC12 knockdown-induced increase in mitotic index. Furthermore, in the predicted structure of TRAPPC12

the N-terminus seems to be unstructured. These unstructured regions have been shown to facilitate bindings that are controlled by post translational modification (Collins et al., 2008; Iakoucheva et al., 2002; Sandhu, 2009) . The importance of five potential phosphorylation sites that reside in the N-terminus of TRAPPC12 was shown through mutations to be responsible for impeding the function of the protein during mitosis. Taken together, this may suggest that the amino terminal domain may have mitotically-regulated interactions with other proteins involved in mitosis or it may interact with the TPR domain, positioning it for a protein-protein interaction.

This study shows that like many mitotic proteins, TRAPPC12 is hyperphosphorylated during mitosis and it needs to be dephosphorylated for cells to exit mitosis. Following phosphorylation shortly after entry of the cells into mitosis, TRAPPC12 dephosphorylation occurs in parallel with a dramatic decrease in cyclin B1 levels. This would be consistent with TRAPPC12 being a substrate for the cyclin B1-CDK1 complex. In further support of this notion, three of the phosphorylation sites that were examined in this study (T107, S109 and S182) conform to a CDK1-cyclin B1 consensus sequence of S/T-P, but further investigation is needed to confirm this hypothesis. Since TRAPPC12 may be phosphorylated during interphase as shown by the lambda phosphatase assay, it is tempting to speculate that TRAPPC12 may dissociate from the TRAPP holocomplex by dephosphorylation, which would allow the protein to function in mitosis. The dissociation from the holocomplex may start during late G2 phase when the Golgi begins to fragment (Lucocq et al., 1987). During mitosis, the hyperphosphorylated TRAPPC12 does not seem to be part of the TRAPP complex as shown by the size exclusion chromatography experiments where the protein shifts to lower molecular size fractions. However, inconclusive results were seen with the TRAPPC12 mutants due to overexpression of the phosphomimetic (5D) and

unphosphorylatable (5A) mutants of TRAPPC12 where no shift in molecular size was detected following colcemid treatment. This suggests that for future experiments stable cell lines that express the TRAPPC12-5A and -5D mutants at normal cellular levels should be established.

A number of facts suggest a strong link between TRAPPC12 and CENP-E during mitosis. The observations presented in this study show that depletion of TRAPPC12 leads to a mitotic arrest which is similar to the phenotype of CENP-E depleted cells (McEwen et al., 2001; Wood et al., 1997; Yao et al., 2000). The TRAPPC12-5D mutant that mimics mitotic phosphorylation is unable to suppress the TRAPPC12-depleted phenotype, which indicates that TRAPPC12 must be dephosphorylated for the cell cycle to progress. Interestingly, mitotic hyperphosphorylation enhances the recruitment of CENP-E to the kinetochore (M. Milev, unpublished results). Moreover, depletion of TRAPPC12 affects the localization of a number of kinetochore proteins, with CENP-E being the most dramatically affected, resulting in chromosome congression defects that activate the SAC (M. Milev, unpublished results).

Overall, the data in this study suggest that TRAPPC12 is a moonlighting protein functioning in two diverse processes. TRAPPC12 is a component of the mammalian TRAPPIII holocomplex during interphase and in mitosis it may be a component of the outer kinetochore. The role of phosphorylation and dephosphorylation of TRAPPC12 needs further investigation. To understand the mechanism of how TRAPPC12 changes functions and how it aids in chromosome congression we first need to explore how it dissociates from the holocomplex and what kinases and phosphatases, if any, are involved in this process.

There have been a number of membrane trafficking proteins that have been found to have other functions. These moonlighting functions occur either in a distinct cellular location or during a different phase of the cell cycle (Royle, 2011). The Rab6A' isoform is associated with the Golgi and trans-Golgi network (TGN) during interphase (Mallard et al., 2002) and it has been implicated in the inactivation of the SAC in mitosis (Miserey-Lenkei et al., 2006). ZW10, involved in trafficking between the ER and Golgi (Hirose et al., 2004), is also a kinetochore component (Kops et al., 2005). AP-2, an adaptor for clathrin mediated endocytosis (CME) (Brodsky et al., 2001), has been shown to interact with BubR1 (Cayrol et al., 2002) and its depletion from cells results in a mitotic phenotype (Boucrot and Kirchhausen, 2007).

To consider a protein as a moonlighting protein it should fulfill an important requirement; it should have one or more unrelated functions that are not due to alternative splicing or gene fusion (Jeffery, 1999). Convincingly demonstrating a moonlighting function is a challenge. Mitotic defects could arise not by the depletion of the protein by siRNA but, rather, as a consequence of altered membrane trafficking. For example, when a cell is depleted of a membrane trafficking protein over a typical 48-72 hour knockdown period, the cell must pass through approximately 3 cell cycles with decreasing amounts of the that protein. Previous studies have shown that the knockdown of TRAPPC12 causes Golgi fragmentation (Scrivens et al., 2011). It is noteworthy that Golgi fragmentation seems to be required for the cells to enter mitosis, controlling the cell cycle progression as a "Golgi mitotic checkpoint" (Colanzi and Corda, 2007). This may suggest that since TRAPPC12 knockdown causes Golgi fragmentation it may indirectly influence mitosis. We do not believe this to be the case for several reasons. First, the effect of the depletion is seen in less then 24 hours after siRNA treatment. Second, the TRAPPC12-5D phosphomimetic mutant is unable to suppress

the increase in mitotic index following TRAPPC12 depletion while the unphosphorylatable TRAPPC12-5A mutant can. Finally, other TRAPP subunits have been shown to cause Golgi fragmentation when depleted from the cell but only depletion of TRAPPC12 is able to cause a mitotic arrest. This suggests that merely fragmenting the Golgi prior to mitosis is not sufficient to induce a mitotic phenotype, and that TRAPPC12 is likely playing a direct role in mitosis.

To mitigate the problems of secondary effects created by the depletion of a protein by siRNA, researchers use different approaches. One of the approaches is the use of small molecule inhibitors. Although they are ideal, there are only few available for membrane trafficking proteins. Another approach recently described as 'knocksideways' is very promising (Robinson et al., 2010). The knocksideways method allows proteins to function normally and then can be removed from their site of action by targeting them to the mitochondria in a rapamycin-dependent manner. This method is currently being tested in our laboratory to further explore the dual roles of TRAPPC12.

A logical question that needs to be addressed following this study is: how does TRAPPC12 switch from one role to the other? One obvious mechanism is that mitotic phosphorylation of TRAPPC12 causes its release from the TRAPP complex. This mechanism is common and is seen in the regulation of GRASP65, a Golgi stacking factor whose mitotic phosphorylation is key for disassembly of the Golgi at mitotic entry (Preisinger et al., 2005; Wang et al., 2003). The number of membrane trafficking proteins that have a proposed mitotic moonlighting function is increasing and this has led to the notion that membrane trafficking and mitosis are intrinsically linked (Scita and Di Fiore, 2010).

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