UNIVERSITAT DE BARCELONA

Dissecting the function of γTuRC subunits in microtubule nucleation and organization

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Memòria presentada per Rosa María Ramírez Cota per optar al títol de doctor per la Universitat de Barcelona

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A mi familia y a José Antonio

“Siempre acabamos llegando a donde nos esperan”

José Saramago

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Abstract

The depletion of MZT1 in human cells causes severe mitotic spindle defects. Depleted cells lack centrosomal γ-tubulin and arrest in mitosis with a monopolar spindle configuration. Similarly, double deletion mutants of the plant MZT1 orthologs GIP1a and GIP1b are embryonic lethal due to abnormal spindle microtubule distribution and chromosome mis-segregation. Moreover GIP1a and GIP1b were shown to localize to active nucleation sites in the interphase cortical microtubule array. MZT1 function is conserved in fission yeast, where it interacts with GCP3, and is an essential component for the recruitment of the γ-tubulin complex to the spindle pole body, the centrosome equivalent, but not for assembly of the γ-tubulin complex. However, the molecular mechanisms underlying these effects remain unknown. The main goal of this project is to study how MZT1 regulates γTuRC to control MT nucleation and organization.

In this work I confirm that in human cells MZT1 is a subunit of the γTuRC and is required for the targeting of the γTuRC to centrosomes and for proper spindle formation. By sucrose gradient fractionation I found that in mammalian cells, MZT1 is not required to assemble the γTuRC. Interestingly, I found that MZT1 is necessary for the interaction of the γTuRC with the targeting factor NEDD1/GCP-WD. While in plants and in fission yeast MZT1 interacts with the N-terminal region of GCP3, I found that in mammalian cells MZT1 interacts with a conserve motif at the N-terminal extension of GCP2, GCP3, GCP5 and GCP6. Furthermore, by immunoprecipitation of FLAG-tagged GCPs MZT1 binding motif mutants, I found that the mutants can be integrated into the γTuRC but lost the interaction with GCP-WD and fail to be targeted to the centrosomes.

To study the role of MZT1 in MT nucleation I performed a MT regrowth experiment with U2OS cells over expressing the CDK5RAP2 nucleation-activating fragment (CDK5RAP2 CM1) and depleted of MZT1. The MT nucleation

induced by CDK5RAP2 was lost upon the depletion of MZT1, suggesting that MZT1 in required for the MT nucleation activation mediated by CDK5RAP2.

In summary MZT1 is required for all γTuRC-dependent functions including centriole duplication. MZT1 binds to a conserved motif present in the extended N-termini of GCP2, GCP3, GCP5 and GCP6, allowing specific recognition of fully assembled γTuRC. Binding of MZT1 primes γTuRC for interaction with the adapter NEDD1/GCP-WD for targeting γTuRC to centrosomes. In addition, MZT1-dependent priming is required for the CDK5RAP2 CM1 domain to activate γTuRC nucleation activity. Thus, by enabling specific recognition of γTuRC by targeting and activation factors, MZT1 spatially controls microtubule nucleation.

Spanish abstract

En las células humanas la depleción de MZT1 provoca graves defectos del huso mitótico. Las células deplecionadas carecen de γ-tubulina centrosomal y presentan una detención de la mitosis con una configuración monopolar del huso mitótico. Del mismo modo, mutantes de deleción dobles de planta MZT1 con sus ortólogos GIP1a y GIP1b son letales para los embriones debido a la anormal distribución de los microtúbulos del huso mitótico y la mala segregación del cromosoma.Además, GIP1a y GIP1b localizan en sitios de nucleación activos de los microtúbulos corticales. La función de MZT1 se conserva en la levadura de fisión, donde interactúa con GCP3, y es un componente esencial para el reclutamiento del complejo de γ-tubulina en el huso polar del cuerpo apical, el equivalente al centrosoma, pero no para el montaje de la γ-tubulina compleja. Sin embargo, los mecanismos moleculares que subyacen a estos efectos siguen siendo desconocidos. El objetivo principal de este proyecto es estudiar cómo MZT1 regula la actividad del γTuRC en la nucleación y organización de los microtúbulos.

En este trabajo se confirmó que en las células humanas MZT1 es una subunidad de la γTuRC necesaria para el anclaje de la γTuRC a los centrosomas, además se ser necesaria para una formación apropiada del huso mitótico. Del mismo modo, por fraccionamiento en gradiente de sacarosa, he encontrado que en células de mamíferos MZT1 no se requiere para el ensamblaje del γTuRC. Curiosamente, he encontrado que MZT1 es necesaria para la interacción de la γTuRC con el factor de orientación NEDD1/GCP-WD. Mientras que en las plantas y la levadura de fisión MZT1 interactúa con la región N-terminal de GCP3, encontré que en células de mamífero MZT1 interactúa con un motivo conservado en la extensión N-terminal de GCP2, GCP3, GCP5 y GCP6. Por otra parte, mediante inmunoprecipitación de las GCPs mutantes del dominino de unión con MZT1, he encontrado que aunque los mutantes se

pueden integrar en el γTuRC pierden la interacción con la NEDD1/GCP-WD sin poder ser dirigidos a los centrosomas.

Para estudiar el papel de MZT1 en la nucleación de MT, he realizado un experimento recrecimiento de MT con células U2OS sobre la expresión de la activación-nucleación de fragmento (CDK5RAP2 CM1) y deplecionado de MZT1. La nucleación de los microtubúlos inducida por CDK5RAP2 se perdió en la depleción de MZT1, lo que sugiere que MZT1 se requiere para la activación de la nucleación microtubular mediada por CDK5RAP2.

En resumen MZT1 es necesaria para todas las funciones γTuRC-dependientes, como la duplicación de centríolos. MZT1 se une a un motivo conservado presente en la N-terminales extendida de GCP2, GCP3, GCP5 y GCP6, lo que permite el reconocimiento específico de γTuRC totalmente ensamblado. La unión de MZT1 al γTuRC "prepara" al complejo para la interacción con el adaptador NEDD1/GCP-WD para la orientación γTuRC a los centrosomas. Además, se requieren esta "preparación" para activar la actividad nucleadora del γTuRC mediada por CDK5RAP2 CM1. Por lo tanto, al permitir el reconocimiento específico de γTuRC por los factores de reclutamiento y los factores de activación, se observa que la MZT1 controla espacialmente la nucleación de microtúbulos.

Abbreviations

AA: Amino acid

AKAP1: A-kinase anchoring protein 1

ATP: Adenosine triphosphate

Augmin: Protein complex required for centrosome-independent microtubule organization

CDK: Cyclin-dependent kinase

Cdk1-Clb5: Cyclin-dependent kinase 1 – B type cyclin 5

CDK5RAP2: Cyclin-dependent kinase 5 regulatory subunit-associated protein 2

CEP: Centrosomal protein

CM1: Centrosomin (Cnn) motif 1

Cmd1: Calmodulin

Cnn: Centrosomin

Co-IP: Co-Immunoprecipitation

CPC: Chromosomal passenger complex

DNA: Deoxiribonucleic acid

EB: End-binding protein

EM: Electron microscopy

ER: Endoplasmic reticulum

G1 phase: Gap 1 phase

G2 phase: Gap 2 phase

GCP: gamma-tubulin complex protein

GDP: Guanosine diphosphate

GFP: Green fluorescent protein

GIP: γ-tubulin interacting proteins

GTP: Guanosine triphosphate

γTuRC: γ-tubulin ring complex

γTuSC: γ-tubulin small complex

HAUS: The human Augmin complex

HURP: Hepatoma upregulated protein

IL-1: Intermediate layers

iMTOCs: Interphase microtubule-organizing center

IP: Immunoprecipitation

KIFs: Kinesin superfamily proteins

Lis1: Lissencephaly 1

MAP: Microtubule associated proteins

MCAK (Mitotic centromere-associated kinesin),

Mps1: Monopolar spindle 1 kinase

MOZART: Mitotic Spindle Organizing Protein Associated with the Ring of γ-tubulin .

MT: Microtubule

MTOC: Microtubule-organizing center

MZT1: MOZART1

MZT2: MOZART2

NEB: Nuclear envelope breakdown

NEDD1: Neural precursor cell expressed developmentally down-regulated protein 1

NME7: nucleoside-diphosphate kinase family member 7

NUDE: Nuclear distribution protein E

NuMa: Nuclear mitotic apparatus protein

PACT: Pericentrin and AKAP450 centrosomal targeting domain

paGFP: photoactivable GFP

PCM: Pericentriolar material

Plk1: Polo like kinase-1

PTMs: post-translational modifications

Ran: RAs-related nuclear protein

Ran-GAP: Ran GTPase activating protein

RanGTP: RAs-related Nuclear protein

RCC-1: (Regulator of chromosome condensation)

RHAMM: hyaluronan-mediated motility receptor

SAC: Spindle assembly checkpoint

siRNA: Silencing RNA

SPB: Spindle pole body

Spc: Spindle pole body component

S phase: Synthesis phase

SPM: Spc110/Pcp1

Stu2: Suppressor of tubulin 2

TACC: Transforming acidic coiled-coil

TEM: Transmission electron microscopy

+TIPs: Plus ending tracking proteins

TOG: (Tumour overexpressed gene)

TPX2: Targeting protein for Xklp2

TSK: TonSoKu associating protein

XMAP215: *Xenopus* Microtubule-associated protein 215

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Introduction

1.1. The microtubule cytoskeleton

Microtubules (MTs) are protein polymers that constitute a highly dynamic macromolecular network to provide support and architecture to eukaryotic cells (Figure 1). Together with actin and intermediate filaments, the MT cytoskeleton plays an important role in the maintenance of cell homeostasis, by supporting cell migration, vesicle and organelle transport and cell division. Additionally, MTs are involved in cilia and flagella formation.

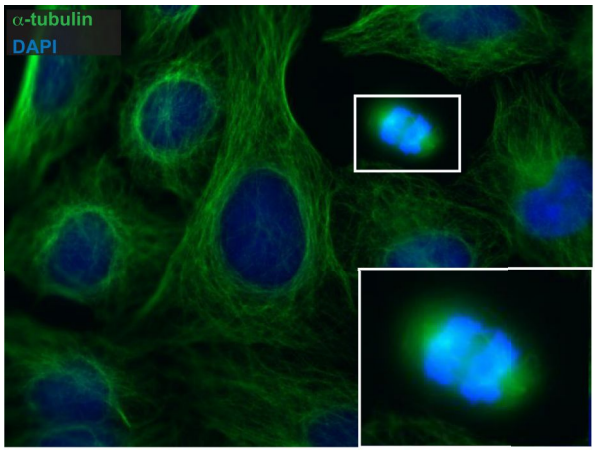

Figure 1. Microtubule organization during interphase and mitosis.

Immunofluorescence microscopy image of HeLa cells fixed and stained with antibodies against α-tubulin (green) to decorate microtubules and DAPI (blue) to label the DNA. Blow up shows a cell in mitosis.

The first reports of MTs from the early 1950's by transmission electron microscopy (TEM) imaging in spermatozoids of *Sphagnum* and in ciliated epithelia from metazoan didn't allow much structural insight due to poor specimen preservation (Manton and Clarke, 1952; Fawcett and Porter, 1954; reviewed in Brinkley, 1997). It wasn't until 1963 when the term "microtubule" was coined (Ledbetter and Porter, 1963). Using the fixation method with glutaraldehyde followed by osmium tetroxide introduced by David Sabatini (Brinkley, 1997; Sabatini et al., 1963). Ledbetter and Porter (1963) were able to obtain improved EM images of MTs in plant cells and from spindle MTs (Figure 2).

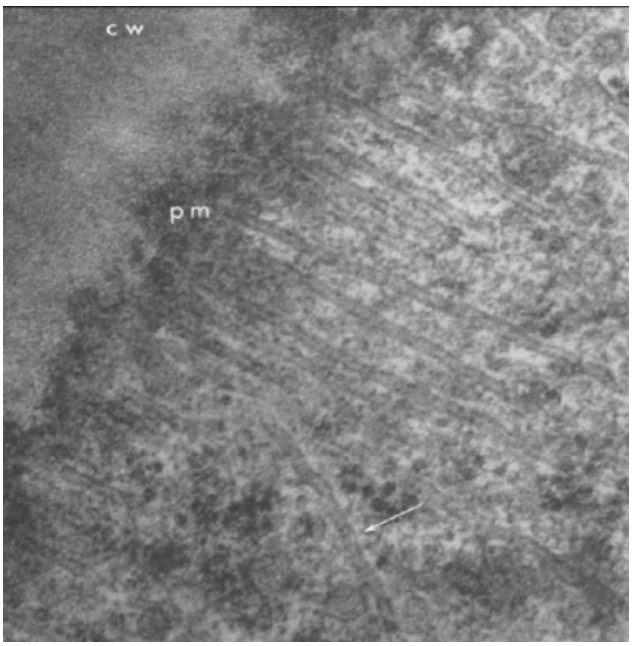

Figure 2. First electron microscopy images of MTs.

EM images from a *Phleum* root cell with the MTs arranged in a parallel orientation, and a few MTs arranged at a 30° degree angle relative to the others (arrow). Cell wall: cw, plasma membrane: pm (From Ledbetter and Porter, 1963).

53 years after the discovery of MTs our knowledge about MT structure and function has greatly increased, but many questions remain unanswered. In this introduction I will summarize the current understanding of MT structure, dynamics, and function, MT-associated proteins that regulate MTs, MT Organizing Centers, and proteins that nucleate new microtubule growth.

1.1.1. Microtubule structure

MTs are hollow cylindrical structures composed of heterodimers of α- and β-tubulin (Figure 3). α– and β–tubulin are proteins of about 55 kDa, share ~40% identity in amino acid (aa) sequence and both bind GTP (Figure 3) (Desai and Mitchison, 1997; Nogales et al., 1998). When the heterodimers are integrated in the polymer the GTP bound at the exchangeable site (E-site) of the β-tubulin subunit can be hydrolyzed to GDP, but cannot be exchanged. This occurs only after MT depolymerization when the GTP binding site on β-tubulin becomes exposed again. GDP at the E-site can be exchanged for GTP, and the heterodimer can be integrated into another polymer. GTP bound to α-tubulin is non-exchangeable (N-site). The hydrolysis of the GTP bound to β-tubulin can

change the conformation of the tubulin heterodimer. GTP-tubulin has a "straight" configuration while GDP-tubulin has a "bent" conformation, but within the polymer GDP-tubulin is forced into the "straight" conformation (Downing and Nogales, 1998; Ravelli et al., 2004).

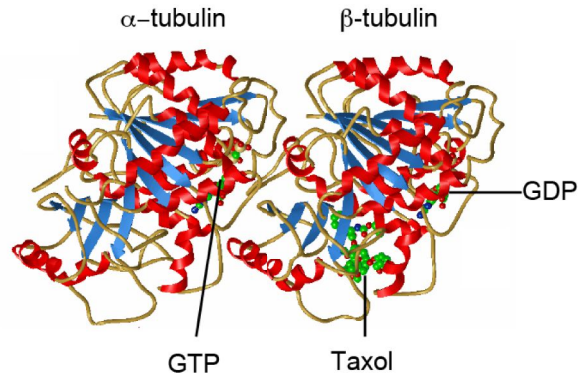

Figure 3. Structure of the tubulin dimer.

Ribbon diagram of the tubulin dimer where α-tubulin is bound to GTP and β-tubulin bound to GDP, and the taxol-binding pocket. (Adapted from Nogales et al., 1998).

The longitudinal interactions of α-β–tubulin form a protofilament and several protofilaments interact laterally to form a hollow cylindrical polymer of 25 nm in diameter (Figure 4) (Kollman et al., 2011). The distribution of the α–β-tubulin along the polymer provides the MT with an intrinsic polarity, with β-tubulin exposed at the relatively dynamic plus-end and α-tubulin facing the minus end, where microtubules are more stable (Figure 4b) (Bergen and Borisy, 1980). Most MTs consist of 13 protofilaments, although *in vitro* MTs assembled from purified mammalian brain tubulin contain 11 to 16 protofilaments (Figure 4a) (Sui and Downing, 2010). The 13 protofilament geometry might be favored *in vivo*, because under these conditions the protofilaments run straight along the MT, allowing the motors proteins to remain on the same face (Kollman et al., 2011). Furthermore, the 13 protofilament geometry might be controlled by the γ-tubulin

ring complex (γTuRC), the major microtubule nucleator in cells (Kollman et al, 2011; Teixidó-Travesa et al, 2012). About 13 molecules of γ-tubulin in the complex provide a platform where α–β-tubulin dimers associate to assemble a 13 protofilament MT (Kollman et al., 2011, 2010; Moritz et al., 2000) (The mechanism that controls MT nucleation will be addressed in section 1.3.1.).

The lateral interactions between the protofilaments deviate horizontally, forming a helical turn along the MT. This means that by following one of the subunits spiraling around the MT for one full turn one will end three monomers up the protofilament from where you started. This configuration is called a "3-start helix" (Desai A. and Mitchison, 1997). Thus all MTs have a *seam*, where one α-tubulin subunit has a weak lateral interaction with a β-tubulin subunit (Figure 4).

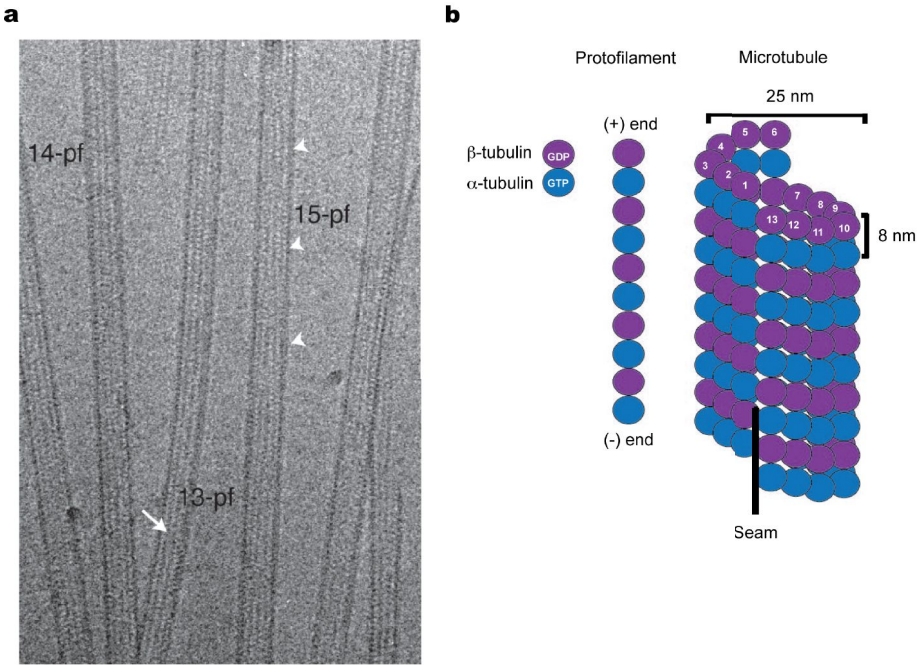

Figure 4. Microtubule structure

(a) Electron microscopy of MTs assembled *in vitro* with 13, 14 or 15 protofilaments (pf). The arrow indicates the 13-pf MT pattern and the arrowheads indicate the 15-pf MT pattern from (Amos and Schlieper, 2005). (b) Organization of tubulins into a 13-pf MT. The dimers associate longitudinally to form a protofilament with an intrinsic polarity with the α-tubulin at the so-called minus end and the β-tubulin at the plus end. 13 protofilaments interact laterally to form a MT of 25 nm in diameter. The protofilaments are slightly deviated so the α-tubulin interacts with the α-tubulin from the next protofilament, except at the seam, where it interacts with the β-tubulin from the next protofilament. Adapted from (Akhmanova and Steinmetz, 2008).

1.1.2. Microtubule dynamics

MTs are highly dynamic structures. MTs assembled *in vitro* from a pool of purified tubulin go through three main phases; a first slow stage named nucleation, followed by a second polymerization stage, where MTs grow faster by the addition of new polymers to the pre-existing MT. Finally MTs can go to a state of depolymerization by the release of dimers at the MT ends. The balance between the addition of dimers at one end and loss of dimers at the other end is called MT treadmilling, which allows the MT to maintain a constant length (Margolis and Wilson, 1978).

MTs can undergo transitions between phases of polymerization and depolymerization, a phenomenon that is termed dynamic instability (Figure 5) (Mitchison and Kirschner, 1984). The transition from polymerization to depolymerization is called catastrophe, and the transition from depolymerization to polymerization is known as rescue. MTs also present pause phases, where neither polymerization nor depolymerization take place. At the structural level the metastable intermediate MT has a blunt end while the depolymerizing end has protofilaments peeling off like a banana peel. The association of proteins that control the transitions between growth and shrinkage phases, promoting rescue or catastrophe, regulates *in vivo* MT dynamics.

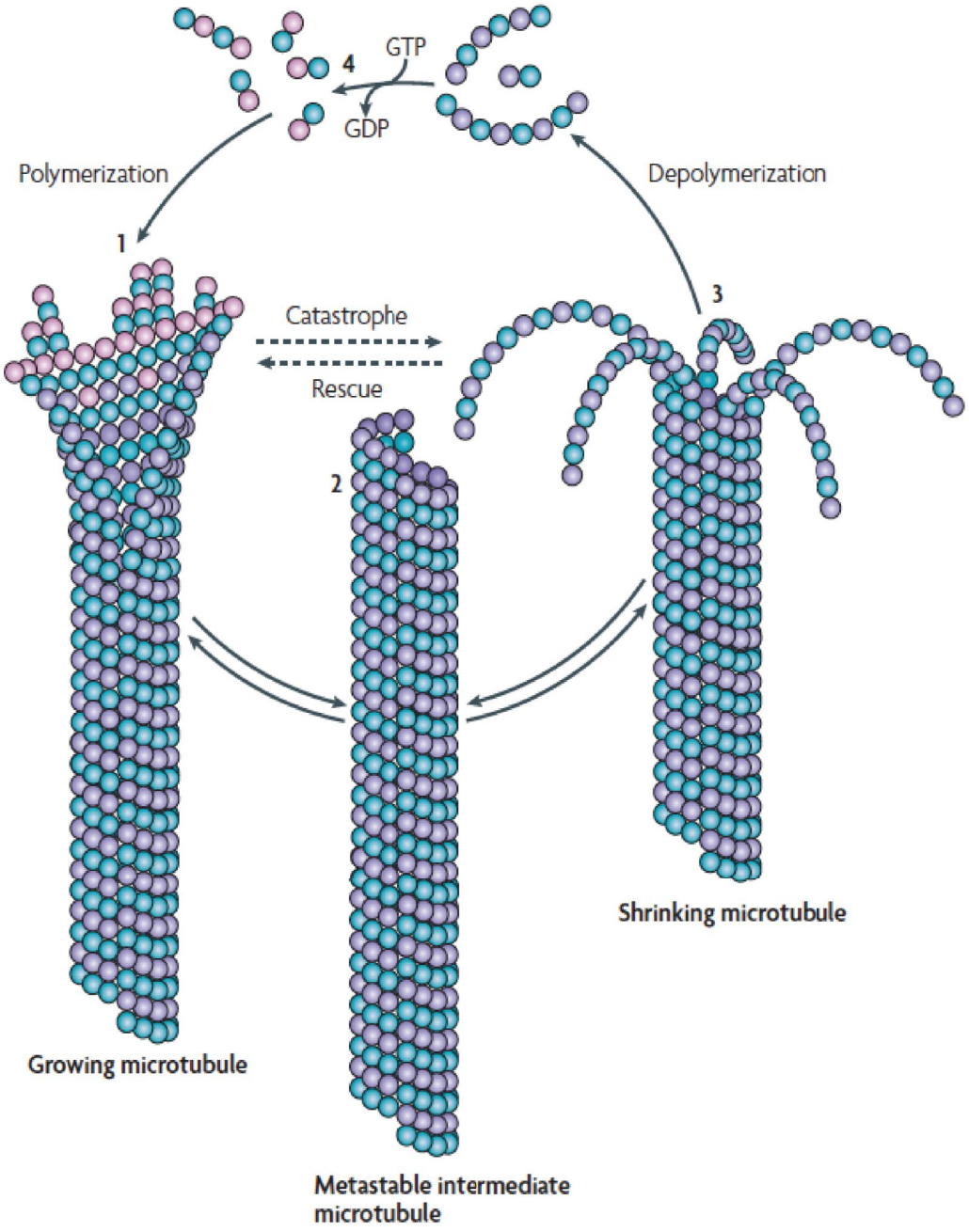

Figure 5. Dynamic instability.

(1) Growing MTs are assembled from a pool of α–β-tubulin. The heterodimers form tubulin sheets that close to assemble the tubular structure with a 25 nm diameter. Growing MTs have GTP-tubulin cap that prevents the microtubule destabilization. (2) Metastable intermediate MTs are characterized by presenting a blunt-end, can pause and then grow (rescue) or depolymerize (catastrophe). (3) Shrinking MTs are unstable MTs presenting curved protofilaments at the shrinking end, like a peeling banana. (4) Free GDP-tubulin dimers exchange GDP for GTP at the β-tubulin subunit, allowing the dimers to enter a second cycle of MT polymerization. Adapted from (Akhmanova and Steinmetz, 2008).

The MT dynamic behavior relies on the individual intrinsic tubulins GTPase activity (Nogales and Wang, 2006). As described before, α-tubulin binds GTP at the N-site (non-exchangeable) located at the monomer-monomer interaction surface of the heterodimer, while the E-site (Exchangeable-site) in the β-tubulin dimer is exposed in the tubulin dimer. When the dimers assemble into a MT the α-tubulin of one dimer acts on the β-tubulin in the adjacent dimer as a GTPase activating protein, leading to GTP hydrolysis on β-tubulin. Since in the polymer the E-site on β-tubulin is non-exchangeable (Downing and Nogales, 1998), this results in a GDP-tubulin body along the MT, making it "intrinsically unstable". However, the continuous addition of dimers to the plus end generates a "GTP-cap" that stabilizes the growing MT plus end and prevents depolymerization (Nogales and Wang, 2006). When the GTP-cap is lost the MT becomes destabilized and undergoes catastrophe (Figure 6).

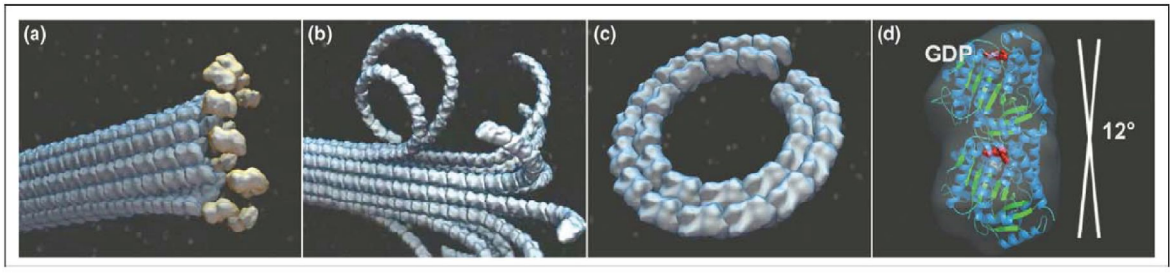

Figure 6. Microtubule catastrophe.

Representation of a MT in catastrophe. (a) The loss of the GTP-cap at the plus end induces the destabilization of the MT. (b) MT in catastrophe with peeling of protofilaments. (c) The peeling protofilaments assemble into a ring-like structure. (d) Bent GDP-dimer that favors MT peeling. Adapted from (Nogales and Wang, 2006).

Apart from regulation of MTs dynamics by GTP hydrolysis and associated proteins, α–β–tubulin are the target of a range of post-translational modifications (PTMs) that control MT functions in cells (Figure 7). Examples of such PTMs are detyrosination by the removal of tyrosine (Y) at the α-tubulin C-terminus in the MT polymer; Δ2-tubulin generation, by the removal of glycine at the C-terminus of

detyrosinated α-tubulin; acetylation by the transfer of acetyl molecule to the Lys40 on α-tubulin, or the Lys252 on β-tubulin; polyglutamylation and polyglycylation, by the addition of glutamic acid (E) or glycine residues (G), respectively, to the γ-carboxyl group of the glutamic acid residues at the C-terminus of the tubulin (Janke and Chloë Bulinski, 2011). These modifications confer stability directly or by promoting the interaction of Microtubule associated proteins (MAPs) and are also believed to have regulatory functions (Janke and Chloë Bulinski, 2011). Furthermore, mutations in tubulin genes lead to neurodegenerative and neurodevelopmental disorders (Chakraborti et al., 2016).

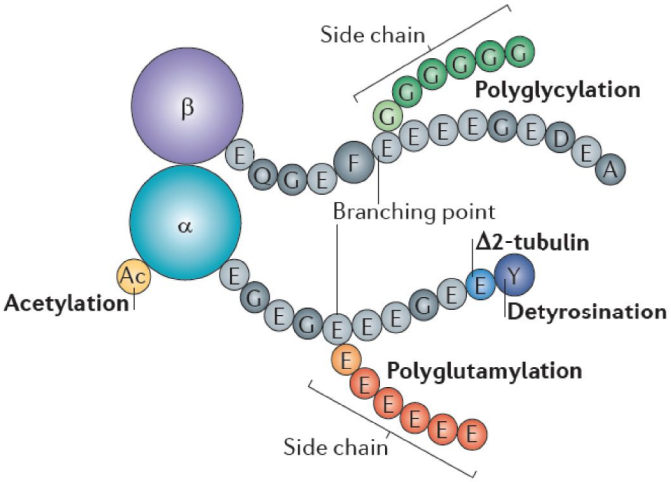

Figure 7. Tubulin PTMs

Schematic representation of the α–β-tubulin modifications. The amino acid chains correspond to the C-terminal domain of α-tubulin (mouse α-tubulin 1A aa 441 to end) and β-tubulin (mouse β-tubulin 2B aa 432 to end). The acetylation of Lys40 is located at the N-terminal domain of α-tubulin. From (Janke and Chloë Bulinski, 2011).

1.2. Microtubule Associated Proteins

As I mentioned above MAPs are proteins than associate with MTs and these associations are controlled by the MTs PTMs and by the intrinsic properties of the MAPs. MAPs can either stabilize the MTs and promote the polymerization or destabilize the MTs and induce depolymerization. Another group of MAPs, the motor proteins, can travel along the MTs to transport vesicles and organelles.

1.2.1. Microtubule stabilizing proteins

Among the stabilizing proteins the best characterized are the tau family, which includes tau, MAP2 and MAP4. Tau organizes parallel MTs into bundles and also promotes MT nucleation and stabilization (Mandelkow and Mandelkow, 1995). MAP4 promotes MT assembly, increases the rescue frequency and reduces the catastrophe frequency (Ookata et al., 1995).

Some MAPs associate exclusively to the growing end of the MT, these are known as +TIPs (Plus end tracking proteins). The +TIPs comprise a wide variety of protein families, with variable size and structure that specifically bind to the MT plus end, providing stability and linking the MT to specify structures such as the cell cortex and the kinetochores (Akhmanova and Hoogenraad, 2015). The first +TIP described was CLIP-170 decorating MT plus ends during polymerization and being lost during pause or catastrophe (Howard and Hyman, 2003). Another well-characterized +TIP is EB1, which associates to GTP-tubulin at the MT plus end, promoting its stability and reducing the catastrophe (Akhmanova and Hoogenraad, 2005; Tirnauer et al., 2003). Purified EB1 and a minimal functional fragment of CLIP-170 (ClipCG12) cooperate to regulate MT plus ends stabilization by the displacement of a stable bound P_i at the MT cap (Lopus et al., 2013). XMAP215 (*Xenopus* Microtubule-associated protein 215) is a +TIP that binds to growing and shortening MTs plus end (Brouhard et al., 2008). Recently it was shown that XMAP215/ch-TOG and the anti catastrophe factor TPX2

(targeting protein for Xklp2) promotes MT nucleation (Wiekzorek et al, 2014). The role of TPX2 and ch-TOG MT nucleation will be addressed in section 1.4.3.

XMAP215 promotes MT assembly in *Xenopus* egg extracts (Gard and Kirschner, 1987) and plays an important role in rapid polymerization and turnover by increasing the MT polymerization rate of tubulin at the plus end, and inducing the rapid depolymerization and reducing the rescue frequency (Vasquez et al, 1994). XMAP215 binds directly MT plus tips and catalyzes the addition of ~25 tubulin subunits *in vitro* (Brouhard et al., 2008). XMAP215 protein family share a coiled coil C-terminal domain and an N-terminal domain composed variable TOG (Tumour overexpressed gene) domains, each TOG domain mediates the interaction with tubulin dimers (Al-Bassam et al., 2006; Brouhard et al., 2008). Furthermore the interaction of TOG with tubulin dimers is enhanced through an interaction with the transforming acidic coiled-coil 3 protein (TACC3) (Gergely et al., 2003, 2000; Lee et al., 2001; Peset et al., 2005). Binding to TACC3 (TD domain) at the C-terminal domain of XMAP215 promotes the recruitment of XMAP215 at the kinetochore and promotes MT elongation during mitosis in *Xenopus* egg extracts (Mortuza et al., 2014). Moreover, the XMAP215 family member, Stu2 (Suppressor of tubulin 2), contributes to the kinetochore-microtubule association, and provides tension stability by either stabilizing or destabilizing attachment depending on the level of kinetochore tension (Miller et al., 2016). Thus XMAP215 plays an important role in chromosome segregation.

1.2.2. Microtubule destabilizing proteins

MT-destabilizing proteins promote MT catastrophe by sequestering α–β-tubulin dimers or by severing MTs. Op18/Stathmin sequesters free tubulin by binding to it and can also bind to the GTP-tubulin at the MT plus end promoting its hydrolysis and resulting in catastrophe (Lu et al., 2014).The MT severing proteins cut MTs into short fragments promoting not only catastrophe, but also inducing new MT growth and release of MTs from cellular structures (Sharp and Ross,

2012). The kinesin I (Kin I) family, is the unique group of the motor proteins (described in the next section) that plays a role in MT destabilization (Desai et al., 1999; Kim and Endow, 2000; Lawrence et al., 2002). One example is the kinI kinesin MCAK (Mitotic centromere-associated kinesin), an ATPase that catalytically depolymerizes MTs at the protofilaments ends (Hunter et al., 2003; Wordeman and Mitchison, 1995).

1.2.3. Motor proteins

Motor proteins control vesicle and organelle transport along MTs, play a role in chromosome segregation during cell division, and support cilia and flagella movement. Motor proteins can move along the MT lattice powered by the hydrolysis of ATP. These proteins are organized into two main groups: minus end directed (retrograde) and plus end directed (anterograde) motors.

Kinesin superfamily proteins (KIFs) are molecular motors that transport cargos in a bidirectional way along MTs. KIFs gathers 15 kinesin families, named kinesin-1 to kinesin 14B (Hirokawa et al., 2009). Most of them move in an anterograde fashion. Kinesin-1 (KIF5) was the first member of the family to be identified. It consists of two heavy chains, each associated with a motor domain and with a light chain (tail domain). The head domain binds to the MTs and the tail domain mediates the binding to the cargo.

KIFs have being implicated in many important processes including spindle assemble, transport of MTs in the axon, sliding of MTs, transport of organelles, mRNA granules, vesicles, and the maintenance of cilia and flagella (Ferenz et al., 2010; Hirokawa et al., 2009; Jolly et al., 2010; Scholey, 2008). KIF motility is regulated in cells by different factors such the tubulin isotype, the PTMs of α–β-tubulin subunits and obstacles along the MT track (Verhey et al., 2011).

Dynein uses MTs to mediate retrograde transport of cargo, spindle positioning, nuclear migration, and motility of cilia and flagella(Cho and Vale, 2012; Vallee et

al., 2004). Dynein is a large protein complex of two large domains called the head or ATPase domain, two intermediate or stem domains and two small subunits that mediate the binding to MTs(Cho and Vale, 2012). Dynein interacts with adaptor proteins like the dynactin complex, Lissencephaly 1 (Lis1) and nuclear distribution protein E (NUDE). Dynactin meditates the dynein-cargo interaction, targets dynein to specific sites in the cells and mediates its activation. Lis1 and NUDE regulate dynein during nuclear and spindle positioning (Kardon and Vale, 2009).

The directional movement of the molecular motors along the MTs is dependent on the intrinsic polarity of the MTs, thus MT organization in cells is crucial to control the transport of cargo such as vesicles and organelles in interphase or post-mitotic cells and of chromosomes during mitosis.

1.3. Microtubule nucleation

In vitro nucleation can be spontaneous above a critical concentration of α- and β-tubulin, but as I described in section 1.1.1, in cells this process requires γ-tubulin, a member of the tubulin superfamily that nucleates MTs (Figure 8) (Kollman et al., 2011; Oakley and Oakley, 1989).

Furthermore, MAPs proteins can further enhance MT nucleation. Together with γTuRC the MAPs MCAK, EB1, XMAP215 and TPX2 contribute to controlling MT nucleation (Wieczorek et al., 2015). While GTP hydrolysis inhibits MT nucleation by destabilizing the newly formed MT plus end, the MAPs control the stability of nascent plus ends (Wieczorek et al., 2015). The release of TPX2 from importins will promote augmin-γTuRC MT nucleation close to the chromosomes and at the spindles (Gruss et al., 2002; Wittmann et al., 2001), simultaneously the catastrophe protein MCAK is inactivated by aurora B (Andrews et al., 2004), MT nucleation can be favored by XMAP215 (Reber et al., 2013). Patronin a MT minus end binding protein protects MTs minus ends against depolymerization by kinby Kinesin-13 (Goodwin and Vale, 2010). CAMSAP3 and CAMSAP2 proteins,

minus end binding proteins, play a role in the regulation of MT anchoring and stability at non-centrosomal MTOCs (Takana et al., 2012). Thus MAPs contribute to spatial and temporal control of MT-nucleation.

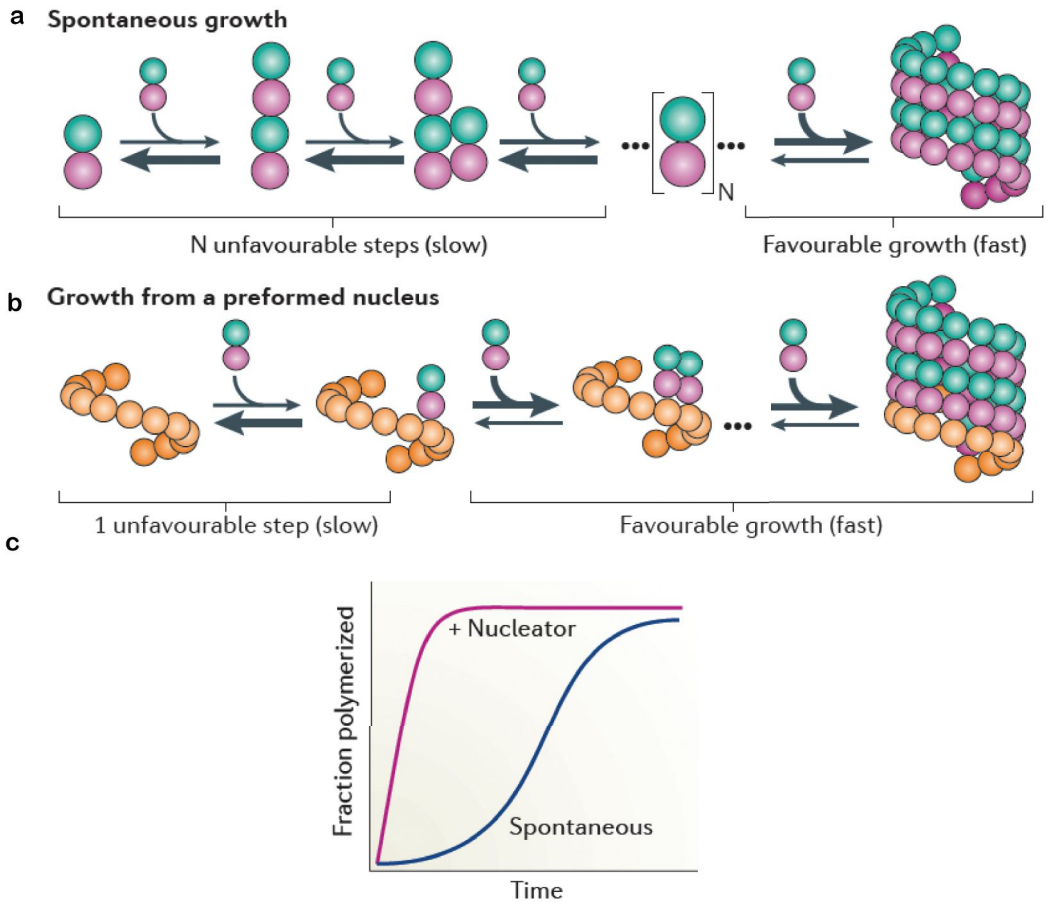

Figure 8. Microtubule nucleation

(a) *In vitro* MTs spontaneously growth from a pool of α–β-tubulin in two phases; a slow phase comprising multiple unfavorable steps, followed by a faster phase, once several oligomers are available (denoted by N). (b) Growth from a preformed nucleus (nucleator) in two steps; one slow step followed by a phase of favorable growth. (c) Graphic representation of spontaneous MT growth compared to growth with a nucleator, where the nucleator accelerates the slow phase. Adapted from (Kollman et al., 2011).

1.4. γ-Tubulin

γ-Tubulin was discovered from a screening of β-tubulin mutation suppressors in *Aspergillus nidulans*. It is a protein of about 50 kDa and a member of the tubulin superfamily, sharing 33% identity with α– and β-tubulin (Oakley and Oakley, 1989). γ-Tubulin localizes to a variety of MTOCs and it is essential for MT nucleation and cell division.

1.4.1. γ-Tubulin crystal structure

The crystal structure of human γ-tubulin bound to GTP-γS (non-hydrolysable GTP analogue) revealed a curved configuration similar to the α–β-tubulin bound to GDP (Aldaz et al., 2005). In budding yeast the binding of GTP to γ-tubulin is essential for the interaction with the α–β-tubulin heterodimers and GTP binding mutants are not viable, fail to nucleate and organize MTs (Gombos et al., 2013). Furthermore, the crystal structure indicates that γ-tubulin can interact laterally with other γ-tubulin molecules using regions that are equivalent to those used by α– and β-tubulin for lateral interactions in the MT lattice (Aldaz et al., 2005).

1.4.2. γ-Tubulin complexes

γ-Tubulin associates with multiple proteins to form two complexes, the γ-tubulin small complex (γTuSC) and γ-tubulin ring complex (γTuRC) (Kollman et al., 2011; Moritz et al., 1998, 1995; Murphy et al., 2001; Teixidó-Travesa et al., 2012; Wiese and Zheng, 2006; C Wiese and Zheng, 2000). In addition to nucleating MTs, the γTuRC stabilizes MTs by capping the minus end (Anders and Sawin, 2011; Christiane Wiese and Zheng, 2000). The EM analysis of purified γ-tubulin complex isolated from *Xenopus* egg extracts revealed that γ-tubulin is associated with several proteins to form a complex with an open ring or lock washer-like shape (Moritz et al., 1998, Zheng et al., 1995). EM of *Drosophila melanogaster*

purified centrosomes reveals several rings of γ-Tubulin with the same diameter as *Xenopus* γTuRC (Moritz et al., 1995). γ-Tubulin in *Drosophila embryo extracts* fractionates as two main complexes, one of ~2.2 MDa named γTuRC and a smaller complex of ~280 kDa named γTuSC (Oegema et al., 1999). *Drosophila* γTuSC is composed of Dgrip84/GCP2, Dgrip91/GCP3, and γ-tubulin (Oegema et al., 1999).

γTuRC is composed of about 13 molecules of γ-tubulin associated with multiple proteins named γ-tubulin complex proteins (GCPs 2-6 in humans) (Kollman et al., 2011; Murphy et al., 2001; Teixidó-Travesa et al., 2012; Wiese and Zheng, 2006). *S. cerevisiae* contains only γTuSC, which is formed by two molecules of TUB4 (γ-tubulin) associated to one molecule of Spc97 (GCP2) and one molecule Spc98 (GCP3) (Figure 9) (Kollman et al., 2008). Multiple γTuSC associate with the Spc72 adapter at the cytoplasmic face or the adapter Spc110 at the nuclear face of the SPB, to assemble into a ring complex, similar to the γTuRC.

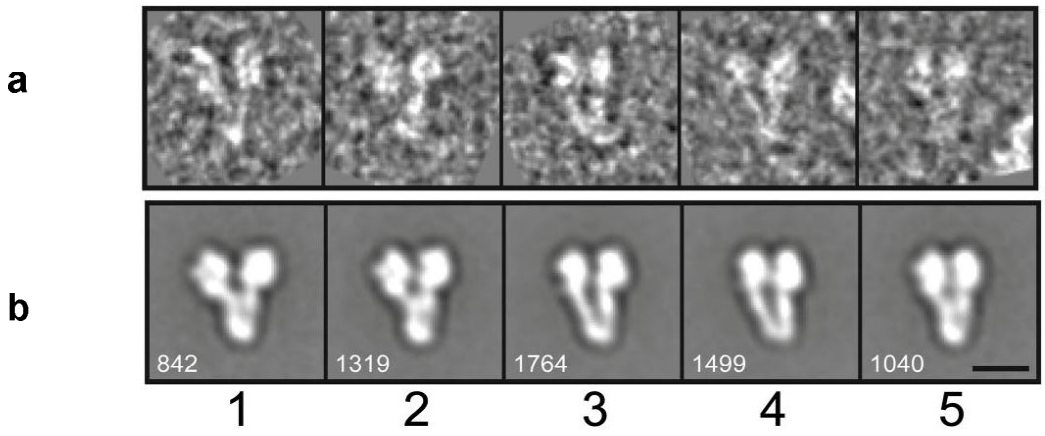

Figure 9. Negative stain of *Saccharomyces* γTuSC.

(a) Individual Y-shaped γTuSC complexes. (b) Reconstruction of the complexes in (a). Adapted from (Kollman et al., 2008).

In the following sections I will describe the structural components of the γTuRC, and the associated proteins that control the targeting and activation activity of the complex.

1.4.3. γTuRC core proteins

GCP2, GCP3, GCP4, GCP5, and GCP6 constitute a protein family and share two-conserved motifs, grip-1 and grip-2 (from γ-tubulin ring protein motif). Grip-1 is located in the N-terminal domain and grip-2 in the C-terminal domain of the GCP core fold (Guillet et al., 2011; Gunawardane et al., 2000; Teixidó-Travesa et al., 2012) (Figure 10).

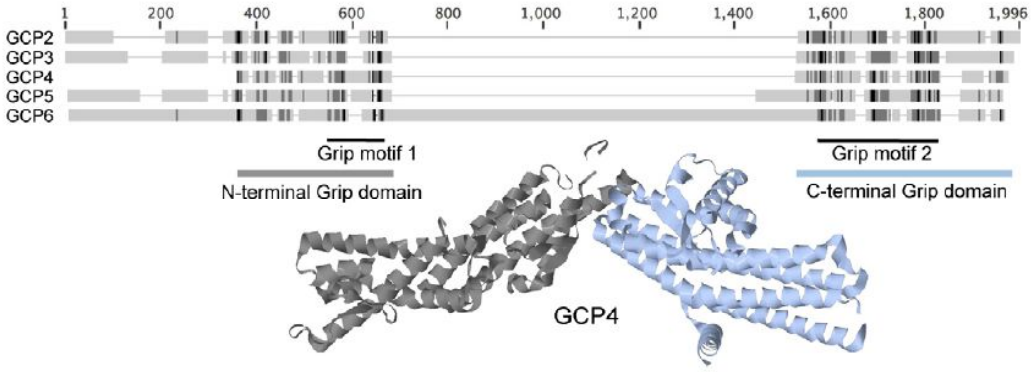

Figure 10. Structural composition of the GCP family.

Aligment of human GCPs 2-6 showing the conserved regions with dark gray. Darker regions indicate a higher conservation degree. Based on the crystal structure of GCP4 the N-terminal and C-terminal grip domains were identified (containing the previously known Grip motifs) and together the two domains constitute the GCP core fold. Adapted from (Teixidó-Travesa et al., 2012).

Insight into γTuRC assembly came from studies of the γTuSC ring structure in budding yeast. The N-terminal domain of Spc110 interacts with GCP3, stabilizing the γ-TuSC complex and promoting oligomerization. In low salt buffer, a purified Spc110-N terminal fragment induces γTuSC oligomerization into a stable filament

in vitro (Kollman et al., 2010). *In vivo* the oligomerization of the γTuSC is controlled during the cell cyle by the phosphorylation of Spc110 both positively and negatively (T. C. Lin et al., 2014). Cryo-EM analysis of γTuSC assembled into a filament showed a structural composition of six and a half γTuSC per turn, with a half-subunit overlapping at the first and the seventh subunits, resulting in 13 γ-tubulin molecules exposed to interact with α/β-tubulin (Kollman et al., 2010) (Figure 11).

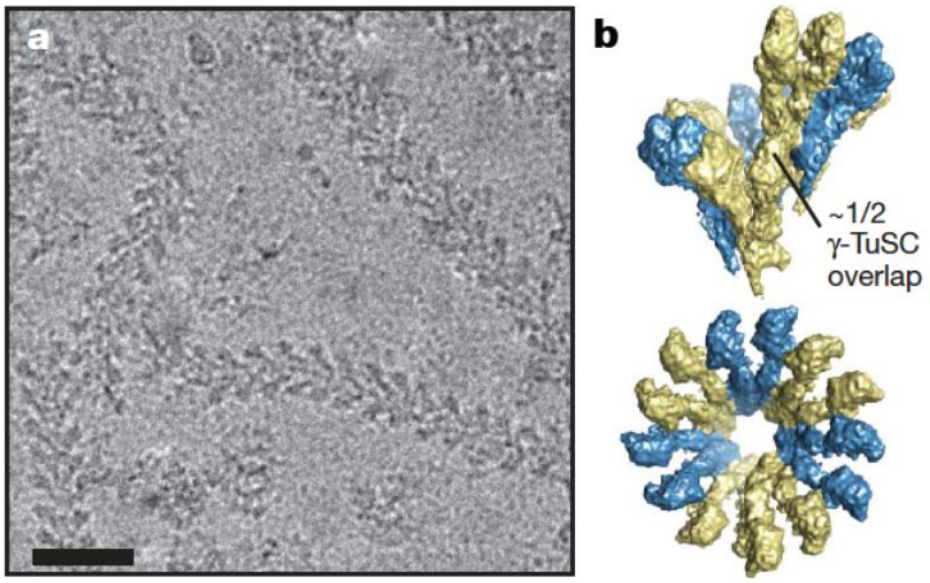

Figure 11. γTuSC oligomer structure

(a) Cryo-electron microscopy image of γTuSC oligomers (Scale bar 50 nm). (b) Side and top view of the γTuSC ring (one turn of the helix). Coloured in yellow and blue are adjacent γTuSCs that interact laterally to form the ring. From (Kollman et al., 2010).

The GCP4 crystal structure provides evidences of strong structure conservation among the GCP family members. Furthermore, the C-terminal domain of GCP4 interacts with γ-tubulin (Guillet et al., 2011), as has been shown for the corresponding region in GCP2 and GCP3 (Choy et al., 2009). Similarly, previous work suggests that the Drosophila GCP5 and GCP6 orthologs can directly interact with γ-tubulin (Gunawardane et al., 2003). These observations lead to the

pseudo-atomic model of the γTuSC oligomer structure, where the C-terminal domain of the GCPs including the C-terminal grip motif is the binding region for γ-tubulin (Figure 12). The N-terminal grip motif might be important for the lateral interactions between the GCPs that occur in this region. Since the C-terminal grip motifs form part of the exposed surfaces of GCP2 and GCP3 they may also be a conserved binging site for other γTuSC interacting proteins (Guillet et al., 2011; Kollman et al., 2010).

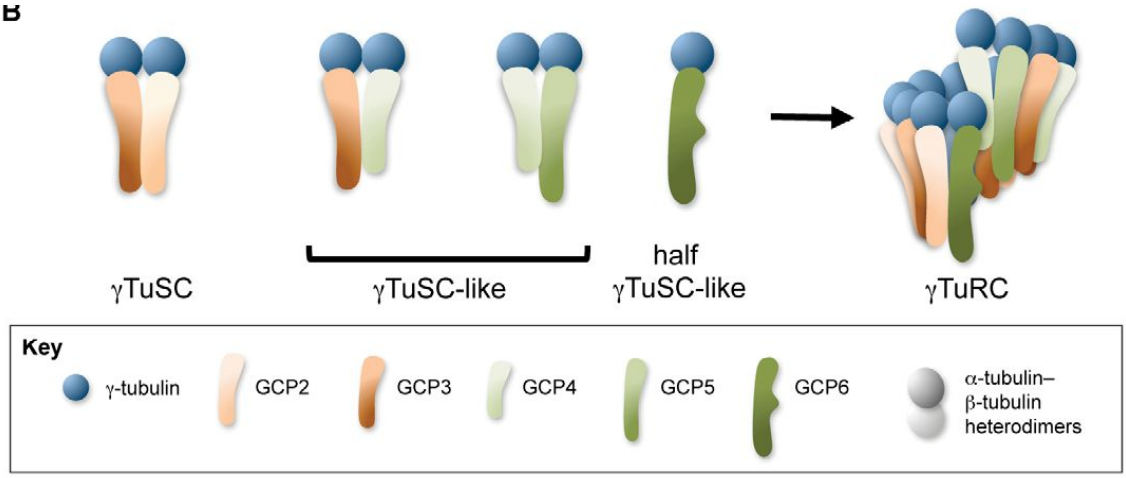

Figure 12. Speculative model of the γTuRC assembly.

Speculative model of γTuRC assembly from γTuSC-like complexes that can be formed from different combinations of the GCPs and γ-tubulin. Adapted from (Teixidó-Travesa et al., 2012).

1.4.4. GCPs and the integrity of the γTuRC

In several organisms the γTuSC components were shown to be essential proteins (Raynaud-Messina and Merdes, 2007), whereas the depletion of the γTuRC-specific GCPs in *Schizosaccharomyces pombe* and *Aspergillus nidulans* doesn not affect the viability of the organism (Anders and Sawin, 2011; Xiong and Oakley, 2009). In *Drosophila* the γTuRC subunits Dgrip75/GCP4, Dgrip128/GCP5 and Dgrip163/GCP6 are required for the assembly of the γTuRC

and are important for centrosome targeting, proper mitotic spindle assembly and progression, but γTuSC is sufficient to support these functions (Vérollet et al., 2006). However, *Drosophila* Grip74/GCP4 and Grip128/GCP5 have an essential role in microtubule anchoring and in germline development, suggesting that these proteins are required in meiosis (Vogt et al., 2006). Unpublished work from our group shows that human GCP4, GCP5 and GCP6 are required for the assembly of the γTuRC (Figure 13) (Teixidó-Travessa, unpublished). The human γTuRC specific GCPs are necessary for proper bipolar spindle formation (Bahtz et al., 2012), and centriole duplication (Figure 14). Interestingly, the depletion of GCP6 causes much stronger mitotic defects than depletion of either GCP4 or GCP5, and the phenotype is comparable to the defects caused by depletion of γTuSC components (Teixidó-Travessa, unpublished). Thus human cells seem to depend strongly on intact γTuRC.

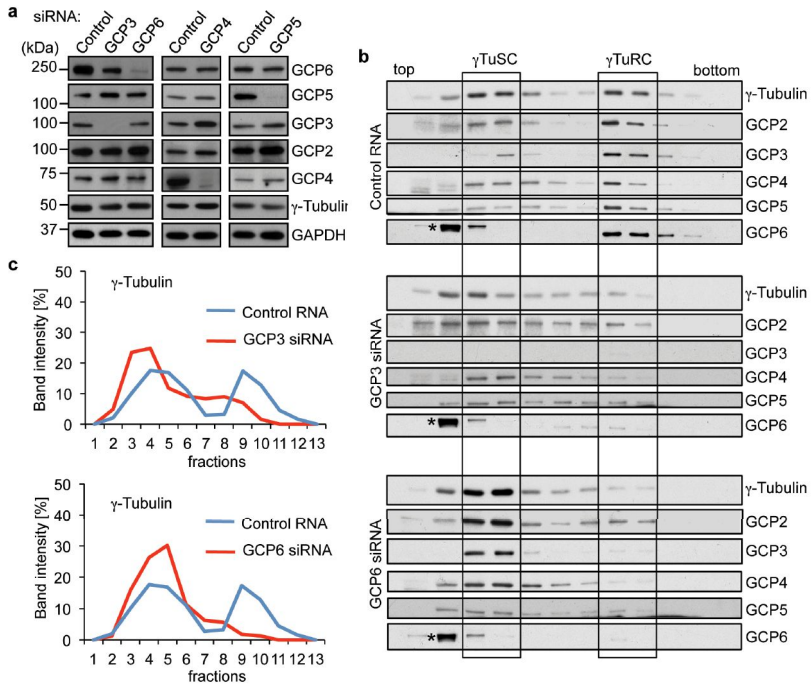

Figure 13. Human GCP3 and GCP6 are required for the assembly of the γTuRC.

(a) Western blot of lysates from HeLa cells treated with siRNA against GCP3, GCP4, GCP5 and GCP6, or control and probed against the indicated proteins of the γTuRC. (b) Western blot of 10-40% sucrose density gradient fractions from HeLa cells lysates treated with siRNA against control, GCP3 or GCP6. (c) Distribution of γ-tubulin band intensity in the Western blot of the sucrose gradient fractions shown in (b). (Neus Teixidó-Travessa, unpublished).

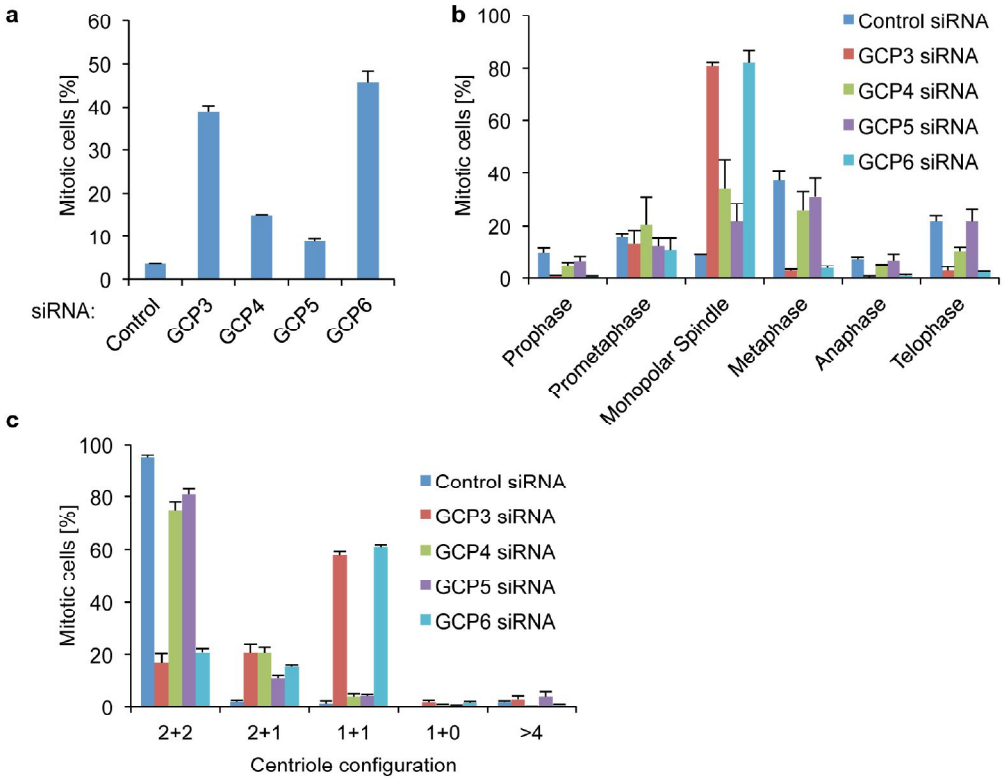

Figure 14. GCPs depletion arrests cells in mitosis and impairs centriole duplication.

(a) Mitotic Index of HeLa cells treated with siRNA against control, GCP3, GCP4, GCP5 and GCP6. Histogram showing percentages of mitotic cells treated as in a (values are the means from n=3 independent experiments; at least 300 cells per condition; error bars: s.e.m.). (b) Histogram showing the distribution of mitotic cells in a, in the different mitotic phases or with monopolar spindle. (c) Histogram of centriole configuration of mitotic cells treated as in a. (Neus Teixidó-Travessa, unpublished).

1.4.5. Mechanism of MT nucleation dependent on the γTuRC: Template vs protofilament model

As I introduced in section 1.1.1.. γTuRC regulates MT nucleation in cells. In this section I will discuss the two models initially proposed to explain the mechanism of γ-tubulin-dependent MT nucleation (Figure 15). One model is based on "template" mediated MT nucleation, where the γTuRC acts as the first helix turn of the microtubule and provides a platform at the minus end of the MT (Zheng et al., 1995). The 13 γ-tubulin molecules in the complex initiate the assembly of 13

protofilaments by longitudinal contacts with γ-tubulin subunits (Zheng et al., 1995). The "protofilament" model suggest that γ-tubulin molecules provide a short segment of a preformed protofilament, were the α–β-tubulin dimers interact laterally to assemble the protofilaments into a MT lattice (Erickson and Stoffler, 1996). Analysis by electron and light microscopy of γTuRC and its interaction with MT minus ends in vitro, has provided more support for the template model, which is now widely accepted (Keating and Borisy, 1999; Kollman et al., 2015, 2011, 2010; Moritz et al., 2000; C Wiese and Zheng, 2000).

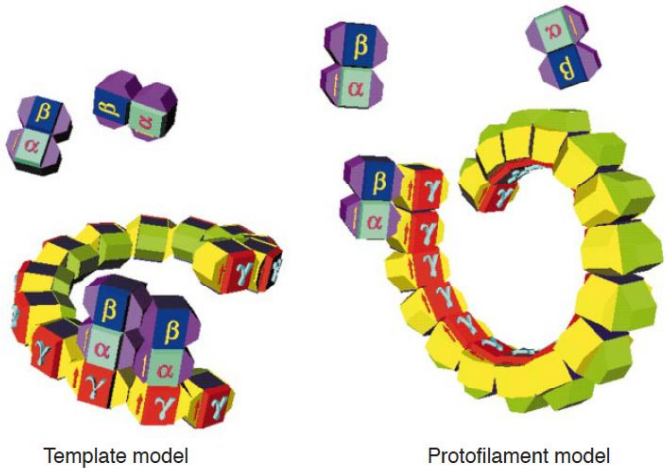

Figure 15. Models for γTuRC-dependent MT nucleation

In the template model the γTuRC provides a helical platform for α-β-tubulin assembly, the 13 subunits of γ-tubulin make longitudinal interactions with α–β-tubulin dimers. In the protofilament model the γTuRC provides a short protofilament, where the α–β-tubulin dimers make lateral interactions with the γ-tubulin. From (Erickson, 2000).

Electron-microscopic tomography images of purified Drosophila γTuRC, demonstrated that the complex has a lockwasher-like structure made of repeated subunits of γ-tubulin (Figure 16), and this ring is associated to the minus end of the MT, supporting the template-mediated MT nucleation (Moritz et al., 2000).

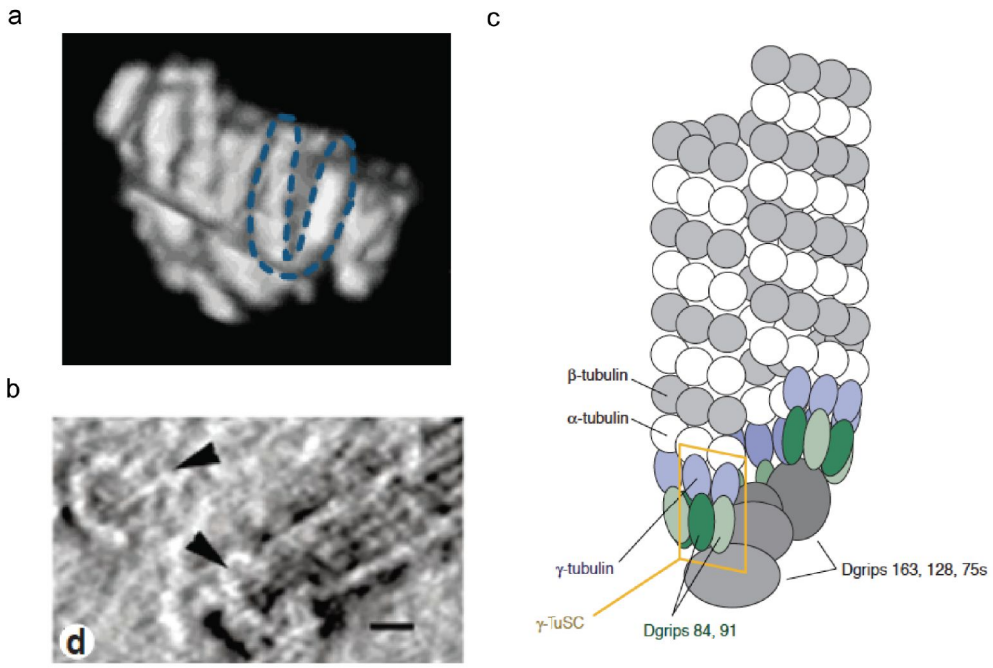

Figure 16. *Drosophila* γTuRC structure and model of MT nucleation by capping the minus end

(a) Representative images of reconstructed isolated *Drosophila* γTuRC obtained by electron-microscopic tomography. (b) Reconstitution of the γTuRC associated to the MT minus end. Scale bar 25 nm, and black arrowheads show the γTuRCs. (c). Model of MT nucleation by the γTuRC as a template model. Adapted from (Moritz et al, 2000).

In vitro γTuRC caps the minus end of MTs, promoting not only MT nucleation, but also regulating MT dynamics of the minus end by blocking loss or addition of tubulin dimers (Figure 17) (Wiese and Zheng, 2000). Consistent with this, the fission yeast γTuC (γ-tubulin complex) stabilizes the MT minus end independently of its nucleation activity in vivo (Anders and Sawin, 2011), suggesting that MT capping might be important to maintain MT stability of MTs released from their nucleation sites (Bellet and Sassone-Corsi, 2010; Keating et al., 1997; Moss et al., 2007).

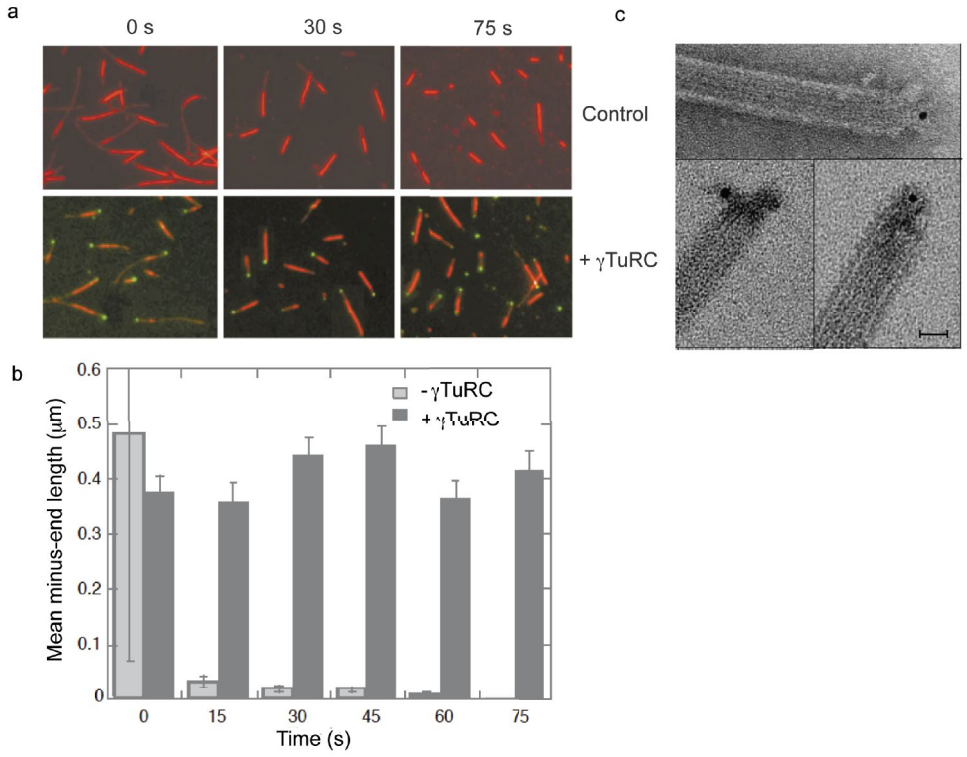

Figure 17. γTuRC caps the MT minus end

(a) Representative images of MTs growing in presence or absence of γTuRC, followed by the induction of shrinking by rapid dilution. Fixing time points are indicated on the top of the panels, microtubules are shown in red and γ-tubulin in green. (b) Quantification of mean values of minus end length in presence of absence of γTuRC. (c) Electron micrograph image of MT associated to gold-labeled γTuRCs. Scale bar 20 nm. Adapted from (Wiese and Zheng, 2000).

1.5. MTOCs

MTs in cells are organized in space and time by specific structures termed microtubule-organizing centers (MTOCs). MTOCs nucleate and anchor MTs, a function that depends on γ-tubulin (Described in chapter 1.4) (Brinkley, 1985; Erickson, 2000; Lüders and Stearns, 2007; Pereira and Schiebel, 1997). MTOCs control MT organization during interphase and mitosis as well as in non-cycling cells. MT orientation depends on the MTOCs, typically MTs are anchored to the MTOCs by the minus end, whereas the plus end explores the cytoplasm. In mitosis the MT minus end is anchored at the spindle poles and the plus end is facing the chromosomes. The main MTOC in animal cells is the centrosome,

which has a equivalent structure, the spindle pole body (SPB), in fungi. Additionally MTs are nucleated at the Golgi apparatus, from lateral sites of preexisting microtubules and in some cells from the cortical membrane. Proteins associated with the γTuRC regulate the targeting of the complex to the variety of MTOCs and also control its nucleation activity.

1.5.1. Centrosomes

The centrosome was described in the late 1800s by Theodor Bovery, as a structure present at the center of the MT network. The centrioles are cylindric structures of about ~0.5 μm length and ~0.2 μm in diameter, composed of nine triplet microtubules arranged in a radial symmetry with γ-tubulin associated in the periphery (Doxsey, 2001; Fuller et al., 1995). Centrioles are arranged in a perpendicular orientation relative to each other, one mother centriole with appendages and a daughter centriole.

The centrosome duplicates once per cell cycle, resulting in two daughter cells, each with a centrosome containing two centrioles at G1. Duplication of centrioles starts at the G1/S transition and continues during S phase. At the end of mitosis the newly formed centrioles become mature before being segregated to the daughter cells at cell division (Fırat-karalar and Stearns, 2014). Although, centrioles are central components of centrosomes, they are not necessary for the centrosome's MTOC activity. Cells that lack centrioles like cells in higher plants and female oocytes can form MTOCs independent of centrioles, and when centrioles are removed from cells by microsurgery or genetically cells can still nucleate MTs and progress through mitosis (Basto et al., 2006; Doxsey, 2001; Hinchcliffe et al., 2001; Khodjakov et al., 2000; Lüders and Stearns, 2007).

The PCM is organized as radial layers of proteins surrounding the centrioles (Fu and Glover, 2012; Lawo et al., 2012; Mennella et al., 2012; Sonnen et al., 2012). CPAP is located at the interface between the centrioles and the PCM, followed by CEP192 and Cep120. CDK5RAP2 (Cyclin-dependent kinase 5 regulatory

subunit-associated protein 2), NEDD1 (Neural precursor cell expressed developmentally down-regulated protein 1) and γTuRC are found at the outer layers. Pericentrin has a extended conformation and is organized radially, with one end proximal to centrioles and other end outer (Lüders, 2012) (Figure 18).

The PCM undergoes dramatic changes during the cell cycle. During the transition from G2 to M the centrosome recruits several proteins. Induced by the activity of mitotic kinases, such as Plk1, several phosphorylation modifications take place and the centrosome increases the size and nucleation activity (Lüders, 2012). The mitotic centrosome is organized in two parts; an inner part with a radial layer organization and an outer extended part organized like a cloud (Fu and Glover, 2012; Lawo et al., 2012; Mennella et al., 2012; Sonnen et al., 2012) (Figure 12 b). In the human mitotic centrosome, the inner part of is organized in a similar manner as in interphase while the outer part contains CDK5RAP2, CEP192, γTuRC and pericentrin (Lawo et al., 2012; Lüders, 2012; Sonnen et al., 2012).

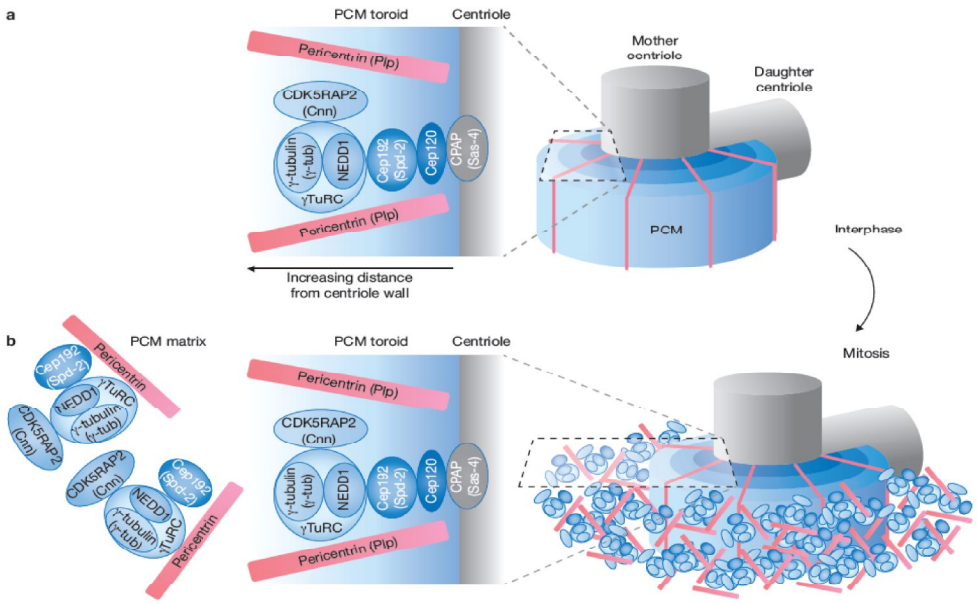

Figure 18. Organization of the PCM.

(a) The interphase PCM matrix is organized into a network of proteins that distributes radially surrounding the centrioles and extended outwards. (b) The mitotic PCM matrix is extended and contains several structural elements and microtubule nucleation proteins. Adapted from (Lüders, 2012).

1.5.2. Spindle Pole Body

The SPB is a multilayered structure embedded in the nuclear envelope. The yeast *Saccharomyces cerevisiae* SPB is the best characterized (Figure 19). It is composed by three main plaques; the external plaque that faces the cytoplasm, the central plaque that is anchored to the nuclear membrane, and the inner plate that is facing the nucleoplasm. Two intermediate layers (IL-1 and IL-2) are located between the external plaque and the central plaque (Bullitt et al., 1997; Jaspersen and Winey, 2004; Moens and Rapport, 1971; O'Toole et al, 1999; Robinow and Marak, 1966 - review by Jaspersen and Winey, 2004). An extension of the central plaque constitutes the half bridge, which controls the SPB duplication (Adams and Kilmartin, 1999; Byers and Goetsch, 1975). The inner and the outer plaque are responsible for the organization of MTs.

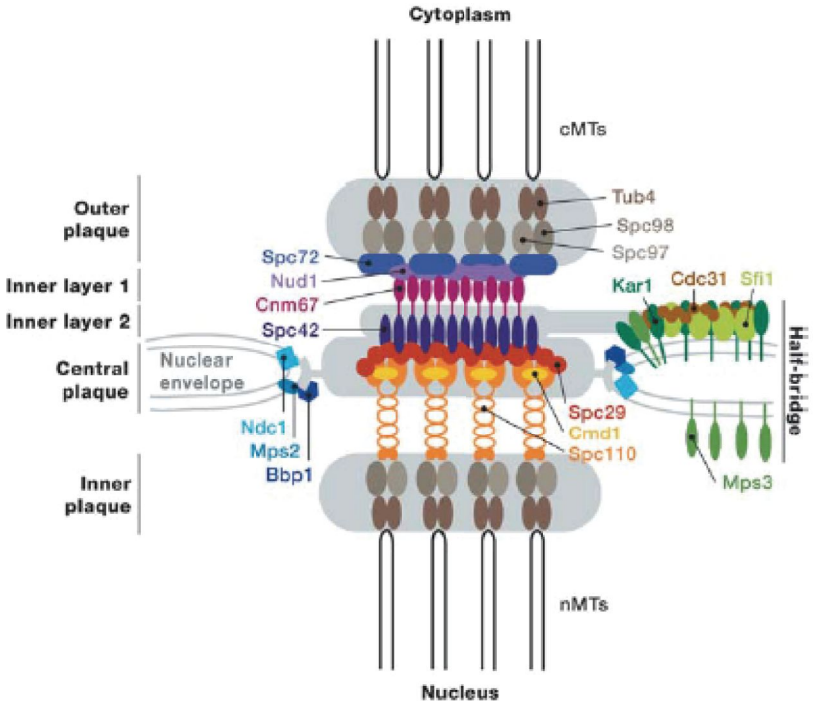

Figure 19. Composition of the yeast SPB.

The Outer and the Inner plaques organize the MTs. The central plaque is embedded in the nuclear membrane, and connected to the half-bridge by an extension of the plaque. From (Jaspersen and Winey, 2004).

Among the described proteins that constitute the SPB in *S. cerevisiae*, Spc110 (Spindle pole body component 110) plays an important role in MT organization and nucleation at the inner plaque of the SPB. The N-terminal domain of Spc110 interacts with the γ-TuSC and through its C-terminal domain interacts with Spc29 and calmodulin (Cmd1) (Elliott et al., 1999; Jaspersen and Winey, 2004; Spang et al., 1996).

The SPB core component Spc42 constitutes the IL2 (Bullitt et al., 1997; Donaldson and Kilmartin, 1996). Through the N-terminal domain Spc42 interacts with Spc29 and Spc110 at the nuclear face of the SPB (Adams and Kilmartin, 1999; Elliott et al., 1999), and with the C-terminal domain interacts with Cnm67 at the cytoplasmic face (Adams and Kilmartin, 1999). Cnm67 interacts with Nud1, a protein required to link cytoplasmic MTs and to exit mitosis (Adams and Kilmartin, 1999; Elliott et al., 1999; Gruneberg et al., 2000). At the cytoplasmic face of the SPB the γ-TuSCs are recruited by Spc72 (Knop and Schiebel, 1997), which is anchored to the SPB by Nud1 (Gruneberg et al., 2000).

1.5.3. Non-centrosomal MTOCs

In fission yeast interphase MTs (iMTOCs) are organized in bundles, in addition to the MTs organized by the SPBs (Bartolini and Gundersen, 2006). Higher plants cells lack centrosomes or a related structure. Instead the microtubules are nucleated from the nuclear envelope, and from pre-existing MTs at the cell cortex (Murata et al., 2005; Wasteneys and Williamson, 1989).

In neurons at an advanced stage of maturation γ-tubulin disappears from centrosomes (Ahmad and Baas, 1995; Baas and Joshi, 1992; Stiess et al., 2010), and MTs are organized as dense non-centrosomal bundles in the cell body as well as in the axon and dendrites (Lyser, 1968). In the axon MTs are organized with the plus end towards the axon tip and the minus end oriented towards the cell body, whereas dendrites have shorter MTs with mixed polarity (Bartolini and Gundersen, 2006). In epithelial cells MTs are nucleated at the

centrosome, and later released and organized in the apical region of the cells (Keating and Borisy, 1999; Keating et al., 1997; Mogensen, 1999; Reilein and Nelson, 2005). In muscle cells (myoblasts), during the formation of myotubes by cell fusion, centrosome proteins are reorganized in clusters around the surface of the nuclear envelope with MTs becoming arranged parallel to the long axis of the myotube (Tassin et al., 1985).

Golgi membranes also play a role as MTOCs in animal cells. The Golgi complex is associated with MT minus ends and can nucleate MTs in interphase cells (Chabin-Brion et al., 2001). The MTs are mostly associated to the cis-Golgi network (Marsh et al., 2001), and this is regulated by the recruitment of γ-tubulin by the protein GMAP-210 (Rios, 2014; Ríos et al., 2004). Golgi nucleation of MTs is necessary for the efficient assembly of the Golgi apparatus in telophase (Maia et al., 2013).

During mitosis MTs are also nucleated and organized at the kinetochores, by the chromatin-mediated nucleation pathway (Heald et al., 1996; Kaláb et al., 2006; Maiato et al., 2004), and within the spindle, by the augmin-dependent nucleation pathway (Goshima et al., 2008; Meunier and Vernos, 2015, 2012). During cell division the chromatin- and augmin-mediated nucleation are activated by RanGTP (Gruss and Vernos, 2004; Meunier and Vernos, 2012; Petry et al., 2013).

A gradient of RanGTP in the proximity of the chromosomes is established by the activity of the guanine nucleotide exchange factor RCC-1 in mitosis (Carazo-Salas et al., 1999). The RanGTP gradient triggers the liberation of spindle assembly factors from importins (Meunier and Vernos, 2012). One important target is TPX2 (introduced in section 1.5.3.). Upon its release from importins, it interacts with RHAMM (hyaluronan-mediated motility receptor/HMMR), GCP-WD/NEDD1 and γTuRC to induce MT nucleation around the chromosomes (Scrofani et al., 2015). MT nucleated close to the chromosomes becomes stabilized around kinetochores by the chromosomal passenger complex (CPC)

through a phosphorylation-dependent mechanism, favoring MT assembly in the kinetochore area (Meunier and Vernos, 2015).

Other proteins controlled by RanGTP are NuMA and HURP (Gruss et al., 2002, 2001; Nachury et al., 2001; Silljé et al., 2006; Wittmann et al., 2001). NuMA allows MT focusing and contributes to the bipolar mitotic spindle (Haren et al., 2006; Merdes et al., 2000, 1996), while HURP contributes to the stability of the K-fibers (Silljé et al., 2006). TPX2 together with the +TIPs EB1 and XMAP215/ch-Tog (introduced in section 1.5.3) contribute to MT nucleation around the chromosomes (Groen et al., 2009).

Nucleation within the spindle is promoted by the octameric augmin complex (HAUS complex in humans), which allows MT nucleation from pre-existing MTs (Goshima et al., 2008; Uehara and Goshima, 2010). The augmin complex facilitates MT nucleation with the help of the γTuRC (Kamasaki et al., 2013; Lecland and Lüders, 2014; Uehara and Goshima, 2010). The newly nucleated MTs are transported with their minus ends leading along pre-existing MTs towards the spindle poles (Lecland and Lüders, 2014). The augmin complex may also play a role in interphase MTs nucleation, since in plants it was reported to participate in generating MTs in the cortical MT array (P. Liu et al., 2014; Murata et al., 2005; Sánchez-Huertas and Lüders, 2015). (For more details on the mechanism of targeting of γTuRC to MTOCs see section 1.5.3).

1.6. Targeting of the γ-tubulin complexes

Several factors associate with γTuRC to regulate where and when MTs are nucleated, such GCP-WD/NEDD1, CDK5RAP2, MOZART1 (MZT1), GCP8/MZT2, NME7, myomegalin, AKP450, augmin and TPX2 (Lin et al., 2014; Teixidó-Travesa et al., 2012). GCP8 might play a role in MT nucleation in interphase(Teixidó-Travessa et al., 2010). Augmin and TPX2 are involved in spindle and chromosomal nucleation by targeting and activating the γTuRC (see section 1.5.3.). GCP-WD/NEDD1, CDK5RAP2 and MZT1 play a role in the

targeting of the complex to specific cellular localizations (Fong et al., 2008; Haren et al., 2006; Hutchins et al., 2010; Lüders et al., 2006). CDK5RAP2 and NME7 (nucleoside-diphosphate kinase family member 7) can activate the γTuRC MT nucleation activity (Choi et al., 2010; T. Liu et al., 2014).

Some γTuRC targeting/attachment factors such as CDK5RAP2 and myomegalin share the conserved centrosomin (Cnn) motif 1 (CM1 motif) (Sawin et al, 2004). Additionally, a subset of proteins like pericentrin, and AKAP450 shares the recently identified Spc110/Pcp1 (SPM motif) (Lin et al., 2014), and the specific centrosomal or SPB-targeting proteins share a PACT domain (from pericentrin and AKAP450 centrosomal targeting domain) (Fong et al., 2009; Galletta et al., 2014; Kawaguchi and Zheng, 2004; Takahashi et al., 2002) (Table 1). Although the exact roles of these motifs are not clear, a recent study in budding yeast has provided new insight into the mechanism that controls the activity of the γTuC (Lin et al., 2014).

Table1. Conservation of γ-tubulin complexes interacting factors in selected model organisms. From (Lin et al., 2014).

Category		Model organism	*Homo sapiens*	*Drosophila melanogaster*	*Arabidopsis thaliana*	*Cryptococcus neoformans*	*Aspergillus nidulans*	*Schizosaccharomyces pombe*	*Candida albicans*	*Saccharomyces cerevisiae*
		Phylum	Chordata	Arthropoda	Streptophyta	Basidiomycota	Ascomycota			
		Type of mitosis	Open	Open/semi-open in syncytial embryo	Open	Semi-open	Semi-open	Closed/ semi-open in meiosis II	Closed	Closed
γ-TuSC		γ-tubulin	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
		GCP2	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
		GCP3	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
γ-TuRC GCPs		GCP4	Yes	Yes	Yes	Yes	Yes	Yes	No	No
		GCP5	Yes	Yes	Yes	Yes	Yes	Yes		
		GCP6	Yes	Yes	Yes	Yes	Yes	Yes		
γ-TuSC receptors	**degSPM–degCM1–PACT**	Pericentrin/D-plp	Yes	Yes	No	Yes	No	No	No	No
		AKAP9	Yes	No		No				
	SPM–CM1–PACT	Spc110/Pcp1/Anpcp1	No	No		No	Yes	Yes	Yes	Yes
	CM1–CM2	CDK5RAP2/Cnn	Yes	Yes		No	No	No	No	No
		myomegalin	Yes	No						
	CM1–MASC	Spc72/Mto1/ApsB	No	No		Yes	Yes	Yes	Yes	Yes
MOZART		MZT1	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
		MZT2	Yes	No	No	No	No	No	No	
γ-TuRC adaptor		NEDD1	Yes	Yes	Yes	Yes	No	No	No	No
Augmin complex		HAUS1	Yes	Yes	Yes	Yes	Yes	No	No	No
		HAUS2	Yes	No	Yes	No	No			
		HAUS3	Yes	Yes	Yes	No	No			
		HAUS4	Yes	Yes	Yes	No	Yes			
		HAUS5	Yes	No	Yes	No	No			
		HAUS6	Yes	Yes	Yes	No	Yes			
		HAUS7	Yes	No	No	No	No			
		HAUS8	Yes	No	No	No	No			
Chromosomal MT nucleation factors		TOG/TOGL	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
		TACC	Yes	Yes	No	No	No	Yes	No	No
		TPX2	Yes	Yes	Yes	Yes	No	No	No	No

As I described in section 1.4.3., the budding yeast SPB proteins Spc110 and Spc72 localize the γTuSC to the nuclear and cytoplasmic side of the SPB, respectively (See section 1.4.3.). The N-terminus of Spc110 directly interacts with Spc98/GCP3 to recruit the γTuSC, inducing the assembly into a ring at the SPB (Kollman et al., 2010; Lin et al., 2014). Spc110 is regulated positively and negatively by phosphorylation throughout the cell cycle (Lin et al., 2014). At S-phase Cdk1-Clb5 kinase and Mps1 kinase phosphorylates Spc110 at the linker region that connects SPM and CM1 motifs, promoting the targeting and oligomerisation of the γTuSC at the SPB. By contrast, the mitotic Cdk1-Clb5 phosphorylates the T18 residue within the SPM to dominantly counteract the activity promoted by S-phase phosphorylation. Spc72, the γTuRC targeting factor at the cytoplasmic face of the SPB, only contains the CM1 motif, so the mechanism of action may differ from Spc110. Furthermore Spc72 cooperates with Stu2 (a Tog-domain protein described in section 1.2.1.) to anchor astral MTs at the cytoplasmic face of the SPB (Usui et al., 2003).

1.6.1. Targeting to centrosomes

In animal cells centrosomal proteins are required for the targeting of the γTuRC to the centrosomes, such as pericentrin (Zimmerman et al., 2004), AKAP450 (Murata et al., 2005) and CDK5RAP2 (Fong et al., 2008). Additionally, MZT1 also is required for the targeting of the complex to the centrosomes, but the mechanism that controls the targeting is not well understood (Hutchins et al., 2010) (See section 1.5.6).

The best-characterized γTuRC-targeting factor in human cells is GCP-WD/NEDD1 (Haren et al., 2006; Lüders et al., 2006). The N-terminal domain of GCP-WD/NEDD1 is required for the binding to the MTOCs and the C-terminal domain mediates the interaction with the γTuRC (Lüders et al., 2006; Manning et al., 2010). The targeting is regulated by phosphorylation of GCP-WD/NEDD1 (Gomez-Ferreria et al., 2012; Johmura et al., 2011; Lüders et al., 2006; Pinyol et

al., 2013; Sdelci et al., 2012; Zhang et al., 2009). Interestingly, neither the CM1 nor SPM motif has been identified in GCP-WD/NEDD1 (Lin et al., 2014).

GCP-WD/NEDD1 is not required to assemble the γTuRC and can localize to centrosomes independently of the γTuRC (Haren et al., 2006; Liu and Wiese, 2008; Lüders et al., 2006; Vérollet et al., 2006). Even tough the γ-TuRC-specific components GCP4, GCP5, and GCP6 were shown to be non-essential for targeting γ-tubulin in organism such as *S. pombe*, *A. nidulas, Xenopus, and Drosophila* (Anders and Sawin, 2011; Venkatram et al., 2004; Vérollet et al., 2006; Xiong and Oakley, 2009), Bahtz et al., (2012) and our unpublished data suggest that GCP4, GCP5 and GCP6 are required for the efficient targeting of γ-tubulin to centrosomes in mammalian cells (Figure 20) (Neus Teixido, unpublished). We found that the depletion of individual GCPs disrupts the interaction of GCP-WD/NEDD1 with the γTuRC, with the strongest effects caused by γTuSC and GCP6 depletions (Figure 20c). Thus, in mammalian cells targeting mediated by GCP-WD/NEDD1 requires an intact γTuRC.

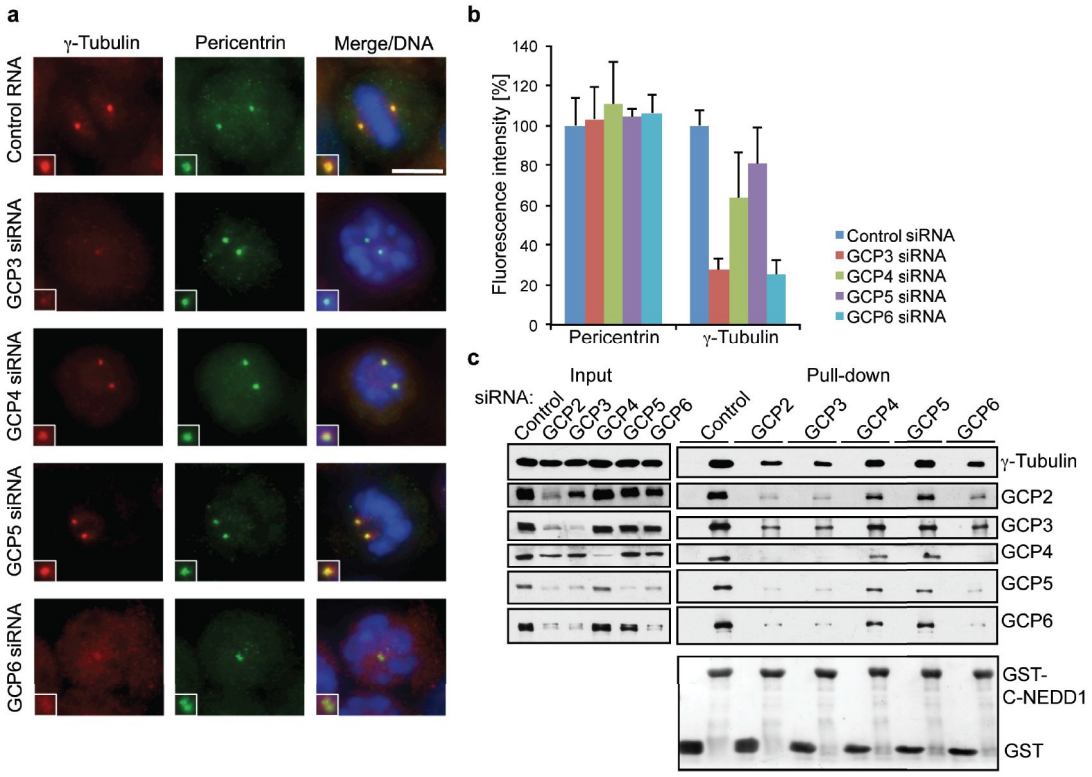

Figure 20. GCPs are required for the targeting of γ-tubulin to the centrosomes.

(a) Representative microscopy images of HeLa cells in mitosis treated with siRNA against GCPs, fixed and stained with γ-tubulin (red) and pericentrin (green) antibodies. DAPI were used to visualize DNA (blue). Insets are magnifications of the centrosomes (Scale bar = 10 μm). (b) Quantification of the fluorescence intensity of γ-tubulin and pericentrin staining at the centrosomes in cells treated as in a. (c) WB of the pull-down of GST-NEDD1 or GST (control) from extract of cells treated as in a (Neus Teixido and Jens Lüders, unpublished).

1.6.2. Targeting to non-centrosomal MTOCs

As I described in section 1.5, MTs are nucleated from a wide variety of cellular structures in addition to the centrosomes, called non-centrosomal MTOCs. In this section I will describe the proteins that recruits γTuRC to those MTOCs.

In human cells, the augmin complex together with GCP-WD/NEDD1 are necessary for the targeting of the γ-tubulin complex to the pre-existing MTs at the spindle, supporting the so-called spindle amplification model (Brugués et al., 2012; Goshima and Kimura, 2010; Haren et al., 2006; Lecland and Lüders, 2014;

Liu and Wiese, 2008; Ma et al., 2010). In *Drosophila*, *Xenopus* and in plants GCP-WD/NEDD1 is required for MT branching on pre-existing MTs through interaction with augmin (Lüders et al., 2006; Vérollet et al., 2006; Zhu et al., 2009). During *Drosophila* oogenesis, GCP4 and GCP5 hare necessary for the formation of central microtubule asters connecting meiosis II spindles in oocytes and cytokinesis in male meiosis (Vogt et al., 2006). GCP6 is required for the targeting of γ-tubulin to the apical zone of mammalian epithelial cells (Oriolo et al., 2007).

In addition to the role in targeting of γTuRC to centrosomes AKAP450 and CDK5RAP2 recruit the complex to the Golgi. AKAP450 localizes specifically to the cis-Golgi compartment (Rivero et al., 2009; Wang et al., 2010). Another protein required for γTuRC Golgi-targeting is myomegalin, which has an N-terminal CM1 motif for binding to the γTuRC and a C-terminal CM2 motif for cellular localization, similarly to CDK5RAP2 (Roubin et al., 2013).

1.7. Regulation of the nucleation activity of the γTuRC

It is not clear how the nucleation activity mediated by the γTuRC is regulated. The best characterized case is the γTuSC from budding yeast, the most basic nucleation complex composed of γ-tubulin, GCP2 and GCP3 (See section 1.4.2.). As I described previously, Spc110 interaction with the N-terminal extension of GCP3 induces oligomerization under low salt concentration *in vitro* (Kollman et al., 2010) and this oligomerization is controlled *in vivo* by cell-cycle dependent Spc110-phosphorylation by Cdk1-Clb5 and Mps1 (Lin et al., 2014). The oligomerization of the γTuSC is a prerequisite to induce the nucleation activity (Kollman et al., 2010). Oligomerization by itself may not be sufficient to induce full MT nucleation activity, because the arrangement of γ-tubulin in the oligomer does not perfectly match the MT symmetry, which may impair nucleation (Kollman et al., 2010). Thus it was suggested that the activation of the complex involves a conformation change that straightens the hinge region in GCP3,

adjusting the position of γ-tubulins in the γTuSC oligomer to better match the symmetry of the MT (Kollman et al., 2011). A recent study *in vitro* revealed that a conformational switch in the complex can be induced experimentally to allow the matching of the γ-tubulin molecules in the γTuSC with the MT minus end, favoring MT nucleation activity (Kollman et al., 2015). In fission yeast Mto1 and Mto2 cooperate to interact with the γTuC in the cytoplasm and mediate MT nucleation (Samejima et al., 2010, 2008). In vivo a minimal functional version of Mto1-Mto2 complex (Mto1/2[bonsai]) is sufficient to activate MT nucleation even without GCP6 (Lynch et al., 2014), suggesting γTuSC is sufficient under these conditions.

In human cells, MT nucleation activity can be controlled by CDK5RAP2. The expression of the CM1 motif of CDK5RAP2 is sufficient to stimulate MT nucleation by γTuRCs in cells and *in vitro* (Choi et al., 2010). Recent work on a *Drosophila* ortholog of CDK5RAP2, Cnn, demonstrated that the phosphorylation by Polo kinase promotes the oligomerization of Cnn promoting MT nucleation in *vivo* and *in vitro* (Conduit et al., 2014). Also, NME7 induces MT nucleation at the centrosomes (Liu et al., 2014). NME7 interacts with γTuRC trough its A and B kinase domains, and mediates the activity of the complex in a kinase-dependent manner (Liu et al., 2014). During mitosis the RanGTP-dependent factors stimulate the nucleation activity in the region close to the chromosomes and the augmin-dependent, branching nucleation mechanism from pre-existing MTs (Gruss and Vernos, 2004; Clarke and Zhang, 2008) (For more details see 1.4.3.). Although a variety of proteins that control γTuRC nucleation activity have been characterized, it is not known if in all cases this regulation acts directly on the γTuRC and whether it involves a conformation change as was observed for budding yeast γTuSC oligomers.

1.8. MOZART1 and MT organization

MZT1 stands for "mitotic-spindle organizing protein associated with a ring of γ-tubulin" and is a protein of only 8.5 kDa that was first described as a member of the human γTuRC (Hutchins et al., 2010; Teixidó-Travessa et al., 2010). Hutchins et al., (2010) identified MZT1 by purification and systematic mass spectrometry analysis of HeLa cells expressing LAP-tagged γ-tubulin, GCP3 and GCP6. As well, MZT1 was found as a member of the γTuRC in human cells, by the purification of LAP-tag γ-tubulin (Teixidó-Travessa et al., 2010). Also, it was shown that MZT1 interacts with the γTuRC by LAP-tagged MZT1 purification and sucrose gradient fractionation. Furthermore, by immunofluorescence analysis it was shown that MZT1 follows the same subcellular distribution as γ-tubulin during the cell cycle (Hutchins et al., 2010).

MZT1 is conserved in eukaryotes, except in the budding yeast. However, MZT1 is present in the genome of other *Saccharomycotina* species that express, like budding yeast, only the γTuSC (Table 1), suggesting a loss of MZT1 during evolution (Lin et al., 2014).

In fission yeast MZT1 is a component of the γTuC and localizes to the spindle pole body and to the spindle (Dhani et al., 2013; Masuda et al., 2013). The yeast and plant MZT1 orthologs were shown to bind directly to the N-terminal region of GCP3 (Dhani et al., 2013; Janski et al., 2012). The MZT1 orthologs in plants, GIP1 and GIP2 localize to the nuclear membrane in interphase and to the spindle in mitosis (Janski et al., 2012; Nakamura et al., 2012). Also, in plants MZT1 localizes to active nucleation sites in cortical MT arrays (Janski et al., 2012) and in yeast the over expression of MZT1 enhances spindle MT assembly (Masuda et al., 2016). Thus MZT1 might play a role in MT nucleation activity mediated by the γTuRC.

In human cells the depletion of MZT1 leads to a mitotic arrest, defects in bipolar spindle formation and loss of γ-tubulin from centrosomes (Hutchins et al., 2010) suggesting that MZT1 has a role in targeting of γ-tubulin complexes. The double

mutation of GIP1 and GIP2 is embryonic lethal and presents impaired development of male gametophytes (Nakamura et al., 2012). The mutants exhibit microtubule disorganization and abnormal spindle polarity and alterations of the nuclear shape (Batzenschlager et al., 2013; Janski et al., 2012). Double mutants of GIP1 and GIP2 causes abnormal nuclear pore complexes distribution and misorganization of the inner nuclear membrane protein AtSUN1 (from *Arabidopsis thaliana* Sad1 and UNC84 domain containing 1) (Batzenschlager et al., 2013). Interestingly, a yeast two hybrid assay showed that plant MZT1 binds to TonSoKu (TSK)-associating protein 1 (AtTSA1), and co-localizes with it at the nuclear membrane, although it is unclear what the function of MZT1 association with the nuclear protein AtTSA1 is (Batzenschlager et al., 2013).

MZT1 is also essential for viability in fission yeast. A MZT1 temperature sensitive mutant presents microtubule disorganization during interphase and mitosis (Dhani et al., 2013; Masuda et al., 2013). A recent study demonstrated, that although the GCPs 4-6 are not essential in yeast, GCP6 deletion together with a MZT1 temperature sensitive mutant shows a synergistic defect in the recruitment of γTuC to the SPBs and for spindle assembly (Masuda and Toda, 2016a). In summary, current evidence suggests that MZT1 is required for both γ-tubulin complex targeting and nucleation activity, but the underlying molecular details are unclear.

1.9. Objectives and strategies of the thesis

1.9.1. Study of the role of MZT1 in the regulation of the γTuRC to control MT nucleation and organization in human cells

Previous studies had demonstrated that MZT1 is a member of the γTuRC required for the proper assembly of MTs during mitosis in plants, yeast and mammalians cells. In yeast and human cells MZT1 is required to recruit γ-tubulin to the centrosomal nucleation centers, but the molecular mechanisms that regulate this process remain unknown. In order to determine how MZT1 affects MT nucleation mediated by the γTuRC in mammalian cells, I focused on the following objectives:

I. Study of the function of MZT1 in cells and in the γTuRC.

II. Characterize the interaction of MZT1 with other proteins.

III. Establish the role of MZT1 in targeting the γTuRC.

IV. Analyze the function of MZT1 in γTuRC nucleation activity.

Materials and methods

2.1. Cloning and plasmids

A full-length cDNA clone of human MZT1 (Addgene plasmid #401221271) was amplified by PCR with the following primers: 5'-TGGCGAGTAGCAGCGGTG -3' and 5'-GCTTGTCATATTTTCAGCAGCC-3'. For expression in human cells, amplified MZT1 was inserted into the plasmid pEGFP-C1 (Clontech, Palo Alto, CA) containing a modified multiple clone site using *FseI* and *AscI* restriction sites. For the expression of Histidine or glutathione S-transferase (GST) fusion proteins in *Escherichia coli*, the full-length open reading frame of MZT1 or N-terminal fragments of GCP2-GCP6 (GCP2 1-506, GCP3 1-552, GCP4 1-347, GCP5 1-713, GCP6 1-710) were inserted into pET28aFA (obtained from Fang lab) using *FseI* and *AscI* or at the pGEX-4X-1 vector (GE Healthcare, Piscataway, NJ) using *EcoRI* and *XhoI* restriction sites. To express pFLAG-GFP the GFP full length was inserted in pCS2+N-FLAG (Addgene). FLAG-GCP3 I93A/L94A/L97A, FLAG-GCP5 I110A/L111A/L114A, FLAG-GCP6 V110/L111A/L114A mutants where generated by site directed-mutagenesis (following the recommendations of QuikChange Site-Directed Mutagenesis). Other plasmids used in this study were pFLAG-GCP2, FLAG-GCP3, pFLAG-GCP5, pFLAG-GCP6 and pGFP-CDK5RAP2 50-90. The following fragments of the N-terminal domain of GCP2-6 where PCR amplified and cloned in pCS2+N-FLAG using *FseI* and *AscI* restriction sites; GCP2 1-506, GCP2 217-506, GCP3 1-552, GCP3 250-552, GCP4 1-347, GCP5 1-713, GCP5 270-713, GCP6 1-710, GCP6 357-710. For expression in yeast, the N-terminal fragments GCP2 1-506, GCP3 1-552, GCP4 1-347, GCP5 1-713, GCP6 1-710 and MZT1 where inserted into *FseI/AscI* restriction sites of the plasmids pGBKT7 and pGADT7 (Clontech, Palo Alto, CA). The mutants GCP3 1-552 I93A/L94A/L97A, GCP5 1-713 I110A/L111A/L114A, GCP6 1-710 V110/L111A/L114A for yeast expression where generated by site directed-mutagenesis. Sequence analysis and alignments were performed with Geneious software (Biomatters; Auckland, New Zealand).

2.2. Cell culture, plasmid and siRNA transfection

Hek293, U2OS and HeLa cells were grown in DMEM (Invitrogen) supplemented with 10% fetal calf serum, at 37°C with 5% CO_2. Cells were transfected with plasmid DNA using calcium phosphate or Lipofectamine 2000 (Invitrogen) according to manufacturers protocol. For calcium phosphate transfection 30 μg of plasmid where mixed with 500 μl HBS (51 mM HEPES, 136 mM NaCl, 5 mM Dextrose, 50 mM KCl, 1.2 mM Na2HPO4, pH 7.1), 30 μl of 2.5 M $CaCl_2$ where added by dropwise. After 20 min incubation, the transfection mixture was added to 70-90% confluence cells seeded in 10 cm dishes (Corning). Transfection was washed after 24 hours.

For siRNA transfection cells where transfected with 100 nM siRNA using LipoRNAimax (Invitrogen). Cells were analyzed after 72h of the transfection. In the case of MZT1 depletion cells were transfected a second time after 48 h. For RNAi mediated depletion the sense sequences are given: MZT1 (C13orf37) 5'-GCUUUAUCAUCGGUUAUUAtt-3' (Ambion ID s54042); GCP2 5'-GAGCUAUGCCUGUACCUAAtt-3' (Ambion ID s21286); GCP-WD 5′-GCAGACAUGUGUCAAUUUA-3′ (According to (Lüders et al., 2006).

To perform microtubule regrowth, dishes containing poly-d-lysine-coated coverslips with U2OS cells were incubated for 30 min in an ice-water bath to depolymerize microtubules. Microtubule regrowth was initiated at 37°C and proceeded for various time periods, followed by methanol fixation.

2.3. Antibodies

Anti-MZT1, anti-GCP2, anti-GCP3, anti-GCP5 and anti-GCP6 rabbit polyclonal antibodies were generated against human His-MZT1 or His-tagged N-terminal fragments of the human proteins (GCP2 1-506, GCP3 1-552, GCP5 1-713 and GCP6 1-710) expressed in ArticExpress cells (Agilent), solubilized in 8 M urea and affinity-purified under denaturing conditions using Ni-Sepharose beads (GE Healthcare). The proteins were used for immunization of rabbits (Antibody Production Service, Facultat de Farmàcia, Universitat de Barcelona, Spain). Anti-

MZT1 specific antibodies were purified by acid elution after binding GST-MZT1 immobilized on Affi-Gel 15 resin (Biorad, Hercules, CA), the anti-GCP2 to GCP6 specific antibodies were affinity-purified using the antigens subjected to PAGE and blotted on membranes.

Other antibodies used in this study: mouse anti-γ-tubulin (GTU-88, Sigma), mouse anti-γ-tubulin (Exbio, Prague, Czech Republic), mouse anti-α-tubulin (DM1A, Sigma), mouse anti-GCP-WD (7D10, Abnova, Walnut, CA), rabbit anti-GCP-WD (Lüders *et al.,* 2006), rabbit anti-GCP6 (AB95172, Abcam, Cambridge, UK), rabbit anti-GCP8 (Teixidó-Travesa et al., 2010), rabbit anti-pericentrin (Lüders *et al.*, 2006), rabbit anti-CDK5RAP2 (Universal Biologicals, Cambridge Ltd), mouse anti-GFP (3E6, Molecular Probes, Carlsbad, CA), rabbit anti-GFP (Torrey Pines Biolabs, Houston, TX), mouse anti-Centrin 3 (H00001070-M01, Abnova, Walnut, CA), mouse anti-FLAG (F3165, Sigma-Aldrich), mouse anti-GAPDH (sc-47724, Santa Cruz Biotechnology, INC), chicken anti-GFP (Aves Labs) Alexa 350- 488- and Alexa 568-conjugated secondary antibodies used for immunofluorescence microscopy were from Invitrogen (Carlsbad, CA), and peroxidase-coupled secondary antibodies for western blotting were from Jackson Immunoresearch Laboratories (West Grove, PA).

2.4. Immunoprecipitation and Western blotting

For immunoprecipitation treated HeLa cells were washed in PBS and lysed (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% NP-40, protease inhibitors) for 10 min on ice. After centrifugation for 15 min at 16,000*g* at 4°C cleared lysates were incubated with antibodies for 1 h at 4°C. Sepharose Protein G beads (GE Healthcare, Piscataway, NJ) were added and the mixture was incubated for an additional hour at 4°C. The beads were pelleted and washed three times with lysis buffer. Samples were prepared for SDS-PAGE by boiling in sample buffer. (0.5M Bis-Tris, 0.3M HCl, 20% glycerol, 8%SDS, 2mM EDTA, 0.06% bromophenol blue, 5% β-mercaptoethanol). Samples were loaded

in an acrylamyde gel (4% for stacking and 10% for separation) and run at 120 V in MOPS buffer (2.5mM MOPS, 2.5mM Tris-base, 0.005% SDS, 0.05mM EDTA) or MES buffer (50 mM MES, 50 mM Tris, 0.1% SDS, 1 mM EDTA) Proteins were transferred to PVDF membranes for 1hour at 60 V in transfer buffer (2.5mM Tris-base, 192mM glycine, 20% methanol). Membranes were blocked in TBS-T (2.5mM Tris-base, 137mM NaCl, 2.7mM KCl, 0.1% tween20) + milk (5%) and probed with antibodies diluted in TBS-T + milk. Membranes were washed with TBS-T between each incubation. ECL Supersignal West Pico reagent (4834080, Pierce) with CL-X-ray Posure film (34091, Thermo Scientific) were used to detect the signal.

2.5. Sucrosse gradient centrifugation

siRNA treated HeLa cell extracts were prepared as described above. 300-400 µl of extract was then loaded on a 4.2 ml 10-40% sucrose gradient prepared in 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA and centrifuged in a SW-55Ti rotor (Beckman, Brea, CA) for 4 h at 55,000 rpm at 4°C. Fractions were collected and analyzed by western blotting.

2.6. Fluorescence microscopy

HeLa cells grown on coverslips were fixed in methanol at −20°C for at least 5 min and processed for immunofluorescence. Fixed cells were blocked in PBS-BT (1x PBS, 0.1% Triton X-100, and 3% BSA) and incubated with antibodies in the same buffer. Images were acquired with an Orca AG camera (Hamamatsu) on a Leica DMI6000B microscope equipped with 1.4 NA 63x and 100x oil immersion objectives. AF6000 software (Leica) was used for image acquisition. For further image processing and quantification of fluorescence intensities ImageJ software was used. Intensities were measured in images acquired with constant exposure settings and were background-corrected.

2.7. Statistic Analysis

Statistical analysis was done using Prism 6 software. Two-tailed, unpaired *t*-tests were performed to compare experimental groups. The results are reported in the figures and figure legends.

Results

3.1. MZT1 is a member of the γTuRC

As a first approach to understanding the function of MZT1 in mammalian cells I described in detail the distribution of MZT1 in cells and also the association with the γTuRC in cells extracts.

3.1.1. Rabbit antibody directed anti human MZT1 production

To further characterize the association of MZT1 with the γTuRC in mammalian cells, I generated a rabbit antibody directed to full length MZT1 produced in bacteria as GST fusion. (Figure 21).

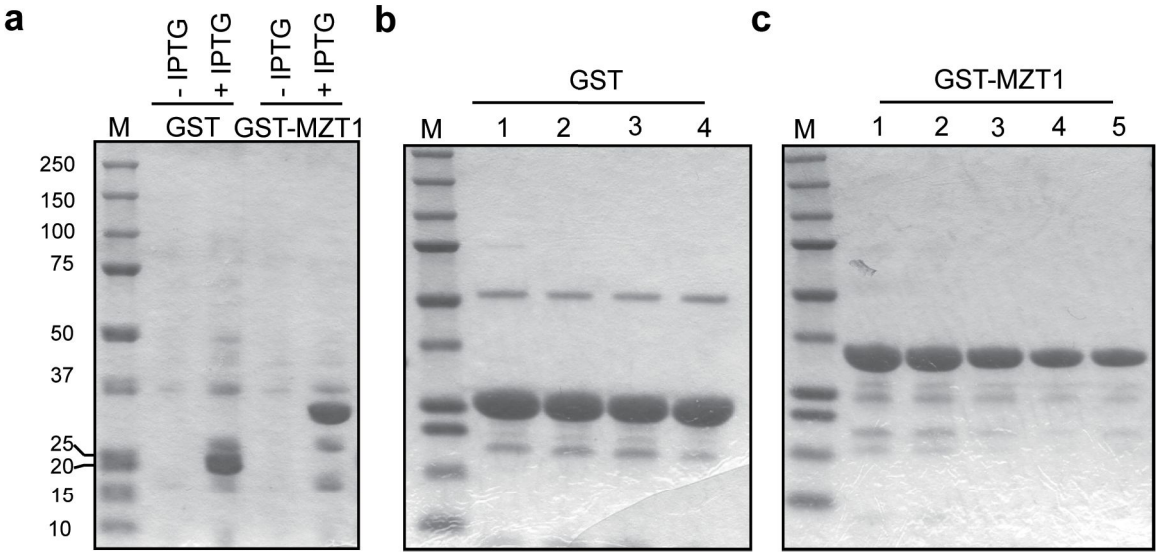

Figure 21. GST-MZT1 protein purification.

GST and GST-MZT1 produced in bacteria analyzed by SDS-PAGE stained with Coomassie blue. (a) IPTG induction of the expression of GST (~25 kDa) or GST-MZT1 (~33 kDa). (b) GST purified fractions 1-4 and (c) GST-MZT1 fractions 1-5. (M: protein molecular mass marker).

I tested the specificity of the antibody by immunofluorescence (IF) of HeLa cells fixed by cold methanol, and by Western blotting (WB) of HeLa cell lysate. Although the antibody recognized centrosomes by IF, I couldn't demonstrate the

specificity (Figure 22), it only recognized over-expressed MZT1 by WB (i.e. GFP-MZT1 or FLAG-MZT1), but not endogenous MZT1 (Figure 23).

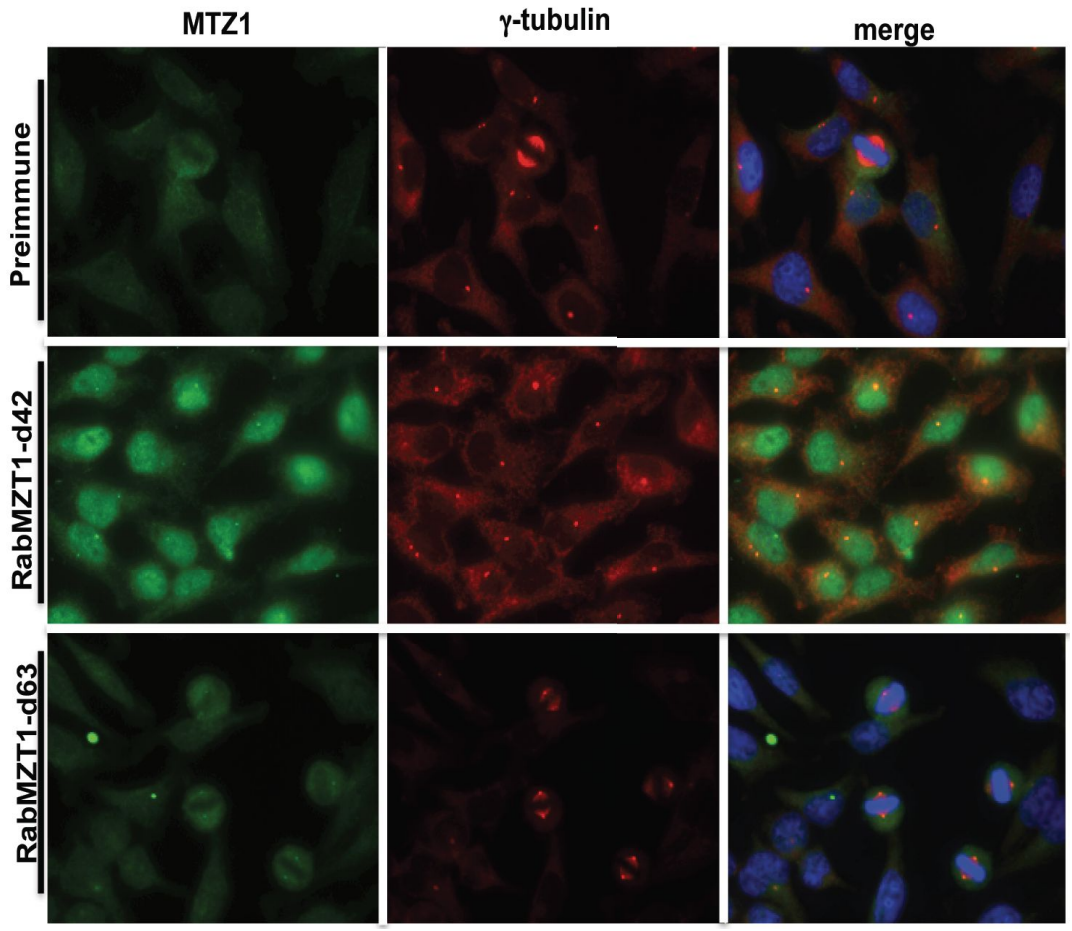

Figure 22. Rabbit anti- GST-MZT1 test.

Representative images of HeLa cells fixed and stained with preimmune serum (control) and two sera from bleedings at day 42 and day 63, after immunization of rabbits with GST-MZT1. Preimmune and immune serum is shown in green, anti γ-tubulin antibody was used to localize centrosoems and spindles (red) and DAPI was used to stain DNA.

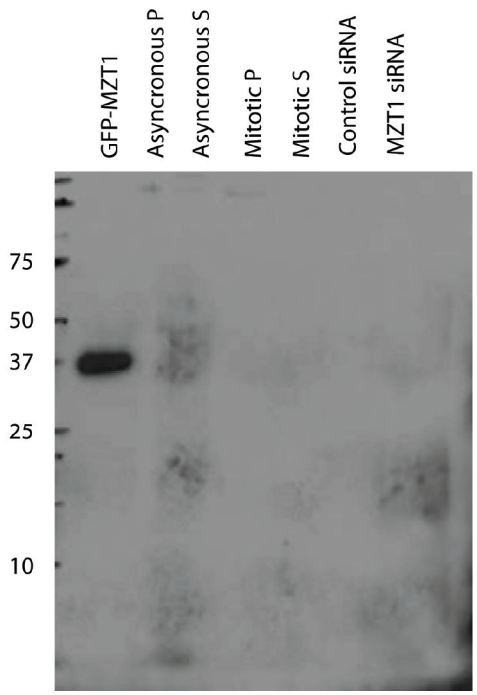

Figure 23. Rabbit anti GST-MZT1 tested by WB.

Rabbit anti GST-MZT1 tested by WB of HeLa cell lysates expressing GFP-MZT1, asynchronous and mitotic pellet (P) and supernatant (S), and cells treated with siRNA anti control or anti MZT1. A band of 37 kDa is shown, corresponding to GFP-MZT1.

Since the GST tag is bigger than MZT1 itself, ~25 kDa and ~8.5 kDa, respectively, I produced a second batch of antibodies directed against the full length MZT1 with a poly-histidine tagged (HIS-MZT1) expressed in bacteria (Figure 24). The preimmune and immune sera were analyzed by IF and WB as before

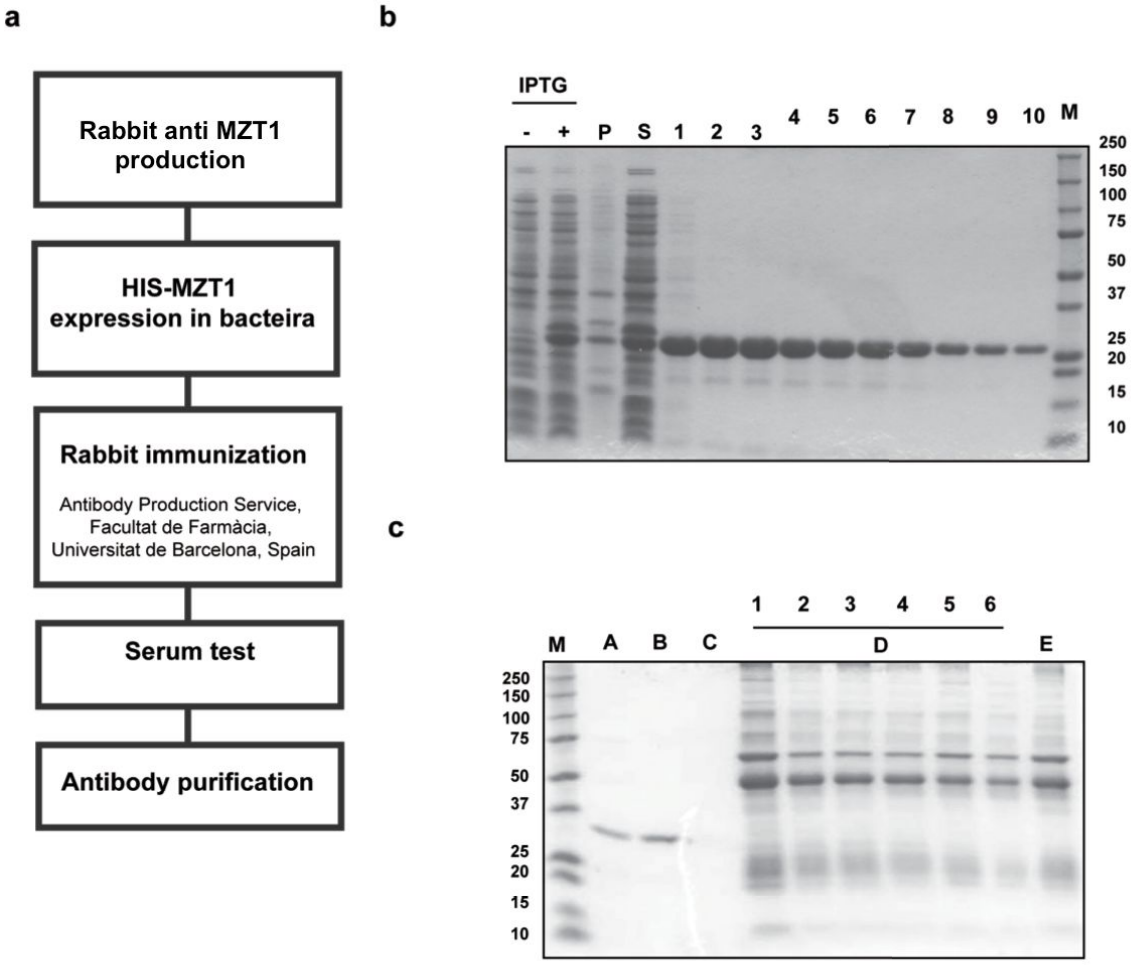

Figure 24. Rabbit antibody directed to full length human MZT1.

Rabbit antibody directed to HIS-MZT1 production strategy. (b) HIS-MZT1 produced in bacteria analyzed by SDS-PAGE stained with coomassie blue. Lane 1: bacterial lysate before the protein induction with IPTG (-IPTG); Lane 2: bacterial lysate after 3h incubation at 37° C induced with 1 mM IPTG induction (+IPTG); Lane 3: insoluble faction (pellet) after cell lysate (P); Lane 4: soluble fraction after cell lysate (S); Lane 5-14: HIS-MZT1 protein fractions collected (1-10); Lane 15: protein molecular mass marker (MW). (b) Antibody purification analyzed by SDS-PAGE stained with Coomassie blue. M: protein molecular marker; A: GST-MZT1; B: GST-MZT1 dialysed with MOPS buffer; C: supernatant after the binding of the GST-MZT1 to the beads; D: Elution of the antibody; E: pool of all the fractions collected.

After the antibody purification, I tested if it was able to detect endogenous MZT1 by Western Blotting of HeLa cell extracts. As shown in figure 23 the antibody detect a band bellow 10 kDa, corresponding to the MZT1 size. I tested several dilutions of the antibody and dilutions of HeLa cell extract to determine the antibody detections limits. The best working condition of the antibody are at a 1:2000 dilution in TBS-tween (5% milk), with a minimum of 50 μg protein lysate loaded. Since MZT1 is a small protein, I tested several transfer conditions to avoid loss of the protein. In summary, MZT1 detection was optimal using transfer buffer without SDS and 60 V for 60 minutes for the transfer. (0.2 μm PVDF membrane was used).

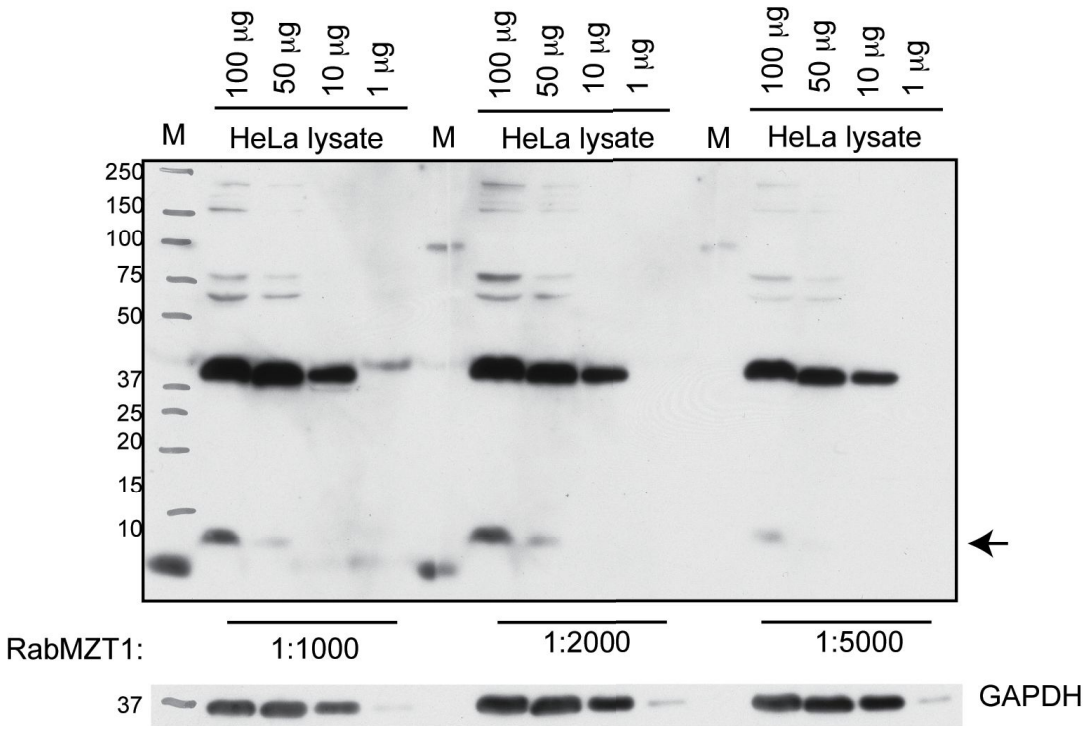

Figure 25. Working dilutions of the rabbit antibody against MZT1 tested by WB.

HeLa cell lysate in different concentrations (100 μg to 1 μg) where loaded and analyzed by Western blot with different dilutions of the purified rabbit anti MZT1, and mouse anti GAPDH was used as a loading control. (Arrow: MZT1).

The specificity of the antibody was probed by treating HeLa cells with MZT1 siRNA, and analyzed by IF and WB (Figure 26). To do the IF analysis of control cells and cells depleted of MZT1, cells were fixed in cold methanol and stained with anti-MZT1 and γ-tubulin antibodies, followed by fluorescence microscopy. I detected a signal of the MZT1 antibody at the centrosome, co-localizing with γ-tubulin in control cells, but after the depletion of MZT1 the signal remained at the centrosomes (Figure 26a, b). Thus, the antibody does not recognize endogenous MZT1 by IF under the tested conditions. The WB analysis of the cell lysates from HeLa cells treated with control siRNA or MZT1 siRNA, indicate that the antibody generated to target endogenous MZT1 is specific for MZT1 (Figure 26c).

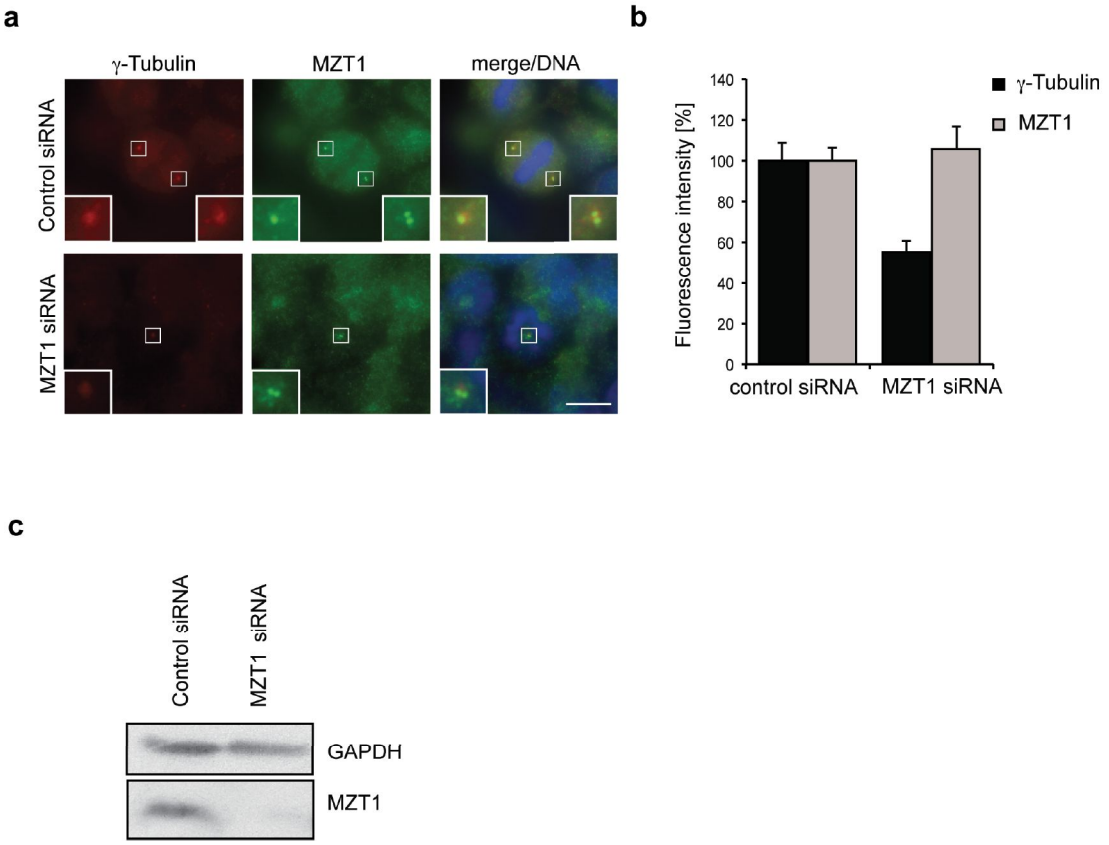

Figure 26. Rabbit antibody against MZT1 specificity test.

(a) HeLa cells treated with control or MZT siRNA, fixed with cold methanol and stained with antibodies against MZT1 (green) or γ-tubulin (red), DNA was stained with DAPI (blue). (b) Quantification of fluorescence intensity at the centrosomes. (c) Cell lysate of HeLa cells treated as in a, and probed for antibody against MZT1 or anti GAPDH as a loading control. (Scale bar 10 μm).

3.1.2. MZT1 interacts with the γTuRC and co-localize with γ-tubulin throughout the cell cycle

After demonstrating the specificity of the antibody I used it to immunoprecipitate endogenous MZT1 from HeLa cells. I confirmed the interaction of MZT1 with different components of the γTuRC, including the targeting factor GCP-WD, contrary to what was found in plants (Nakamura et al., 2012) (Figure 27a). Association of MZT1 with γTuRC was further confirmed by demonstrating cofractionation of MZT1 with γTuRC in sucrose gradients (see section *3.3*). Unfortunately the antibody I generated was not functional for immunofluorescence staining of MZT1, as was demonstrated in the previous section (Figure 26). In order to describe the distribution of MZT1 in cells, I generated a GFP-MZT1 plasmid and transfected this construct into human U2OS cells. By immunofluorescence microscopy of cells expressing GFP-MZT1 I found that MZT1 co-localizes with γ-tubulin throughout the cell cycle (Figure 27b, c). During interphase MZT1 localizes to the centrosomes, in mitosis to centrosomes and to the microtubules of the spindle. Additionally, in telophase and at cytokinesis MZT1 localizes to the MTs at the midbody region (Figure 27b). These results indicate that in mammalian cells MZT1 is a component of γTuRC and colocalizes with the complex throughout the cell cycle.

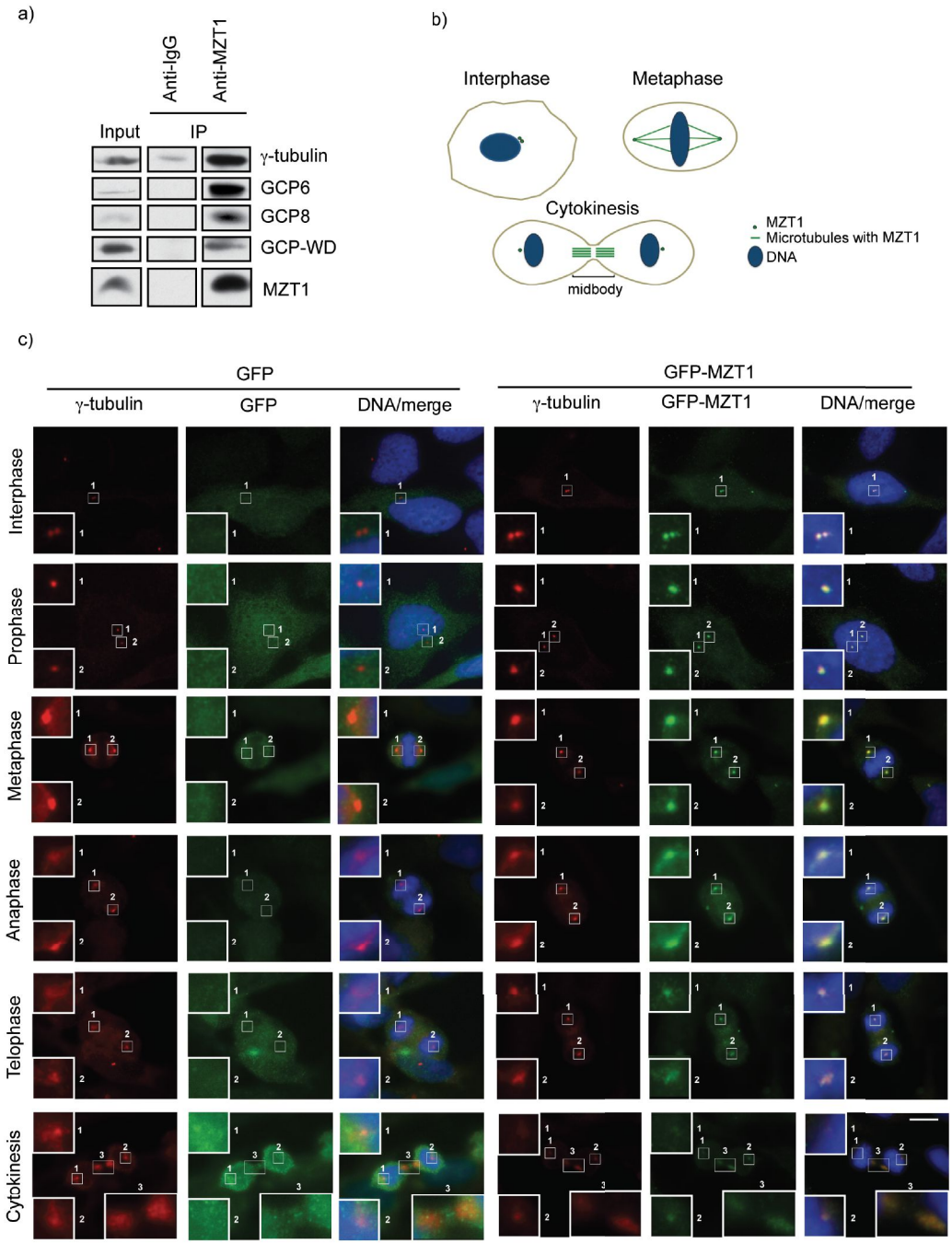

Figure 27. MZT1 interacts with the γTuRC and co-localizes with γ-tubulin through the cell cycle.

(a) Extracts of HeLa cell were immunoprecipitated with control IgG or MZT1 antibody and samples were analyzed by SDS-PAGE and immunoblotting with antibodies for GCP8, GCP6, γ-tubulin, GCP-WD and MZT1 confirming the interaction of MZT1 with the γTuRC. (b) Schematic representation of MZT1 distribution in cells throughout the cell cycle. (c) Immunofluorescence microscopy images of U20S cells transiently expressing GFP or GFP-MZT1 fixed after 72 hours of transfection and stained with γ-tubulin (red) and GFP (green) antibodies; DAPI was used to stain DNA (blue). Insets No.1 and No. 2 are magnifications of the centrosomes, and No. 3 are magnifications of the midbody (Scale bar = 10 μm).

3.1.3. MZT1 over-expression stabilizes the γTuRC

Since the precise role of MZT1 in the γTuRC was unknown, I studied the stability of γTuRC in cells over-expressing MZT1. I subjected extracts of HeLa cells expressing GFP or GFP-MZT1 to fractionation on 10-40% sucrose gradients. The fractions collected were analyzed by Western blotting for different components of the γTuRC. I found that GFP-MZT1 was broadly distributed throughout the gradient, including the fractions corresponding to the γTuSC and the γTuRC, in agreement with results reported by Hutchins et al., 2010. Interestingly, even though most γTuRC proteins peaked in the same fractions as in control extract, their overall distribution indicated a slight shift towards higher molecular weight fractions compared to the fractionation profile in control extract (Figure 28a, b). This observation suggested that MZT1 might promote stabilization of the γTuRC and/or induce a change in conformation or composition.

In order to determine if the over-expression of MZT1 affected the recruitment of γTuRC to the centrosome, I expressed GFP-MZT1 in U2OS cells. 72 hours after transfection of GFP or GFP-MZT1 I fixed the cells with cold methanol, and stained the cells with antibodies for γ-tubulin and GFP. I then quantified the intensity of centrosomal γ-tubulin staining in cells expressing GFP or GFP-MZT1 (Figure 28c). The γ-tubulin intensity in centrosomes of cells in interphase was 2.5 fold increased in cells expressing GFP-MZT1 compared to control, but this strong effect was not observed in mitosis (Figure 28d).

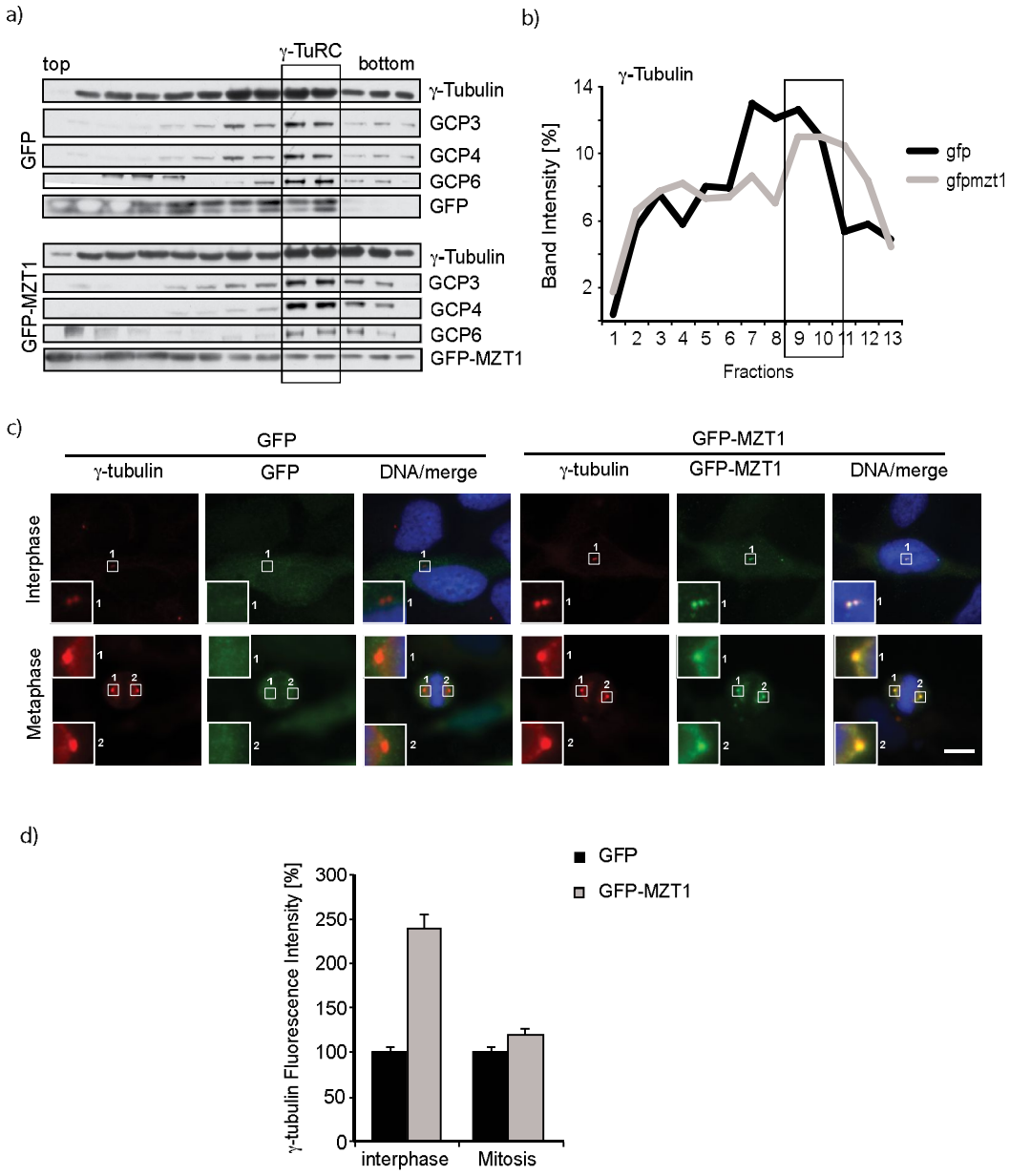

Figure 28. MZT1 overexpression stabilizes the γTuRC.

(a) Cell extracts of HeLa expressing GFP or GFP-MZT1 were subjected to centrifugation through a 10-40% sucrose density gradient, 13 fractions were collected and analyzed by Western blotting with antibodies against γTuRC proteins and GFP, showing that MZT1 overlaps with the γ-tubulin fractions. (b) Distribution of γ-tubulin in the sucrose gradient indicating that GFP-MZT1 expression induces a shift to higher molecular weight fractions. (c) Representative images of U2OS cells expressing GFP or GFP-MZT1 in interphase or metaphase; γ-tubulin (red) and GFP (green) antibodies; DAPI was used to stain DNA (blue). Insets are magnifications of centrosomes. Scale bar = 10 μm. (d) Quantification of γ-tubulin fluorescence intensity at the centrosomes in interphase and cells in metaphase treated as in c, the centrosomal signal measured in control cells was set to 100%. Mean values are blotted as percentages of intensities in control cells (error bars s.e.m.; data from n=40 centrosomes). (e) Quantification of γ-tubulin fluorescence intensity at centrosomes in metaphase of cells treated as in c and analyzed as in d.

3.2. MZT1 is required for mitotic progression and centriole duplication

To further characterize the function of MZT1 in mammalian cells I depleted MZT1 from HeLa cells and analyze the cells for cell cycle defects and stability of the γTuRC. After two consecutive transfections with MZT1 siRNA the mitotic index increased to ~30% compared to ~4% in control cells (Figure 29b), and ~60% of MZT1-depleted mitotic cells presented a monopolar spindle (Figure 29c, d). The monopolar spindle phenotype occurs when the centrosomes fail to separate, giving rise to spindle microtubules that grow from a single pole. Alternatively, monopolar spindles can be the result of centriole duplication defects, leading to cells entering mitosis with a single centrosome. To test if MZT1 affects centriole duplication I quantified the centriole configurations in mitotic cells. I found that MZT1 depletion impaired centriole duplication leading to a reduction in the number of centrioles in >50% of the cells (Figure 30).

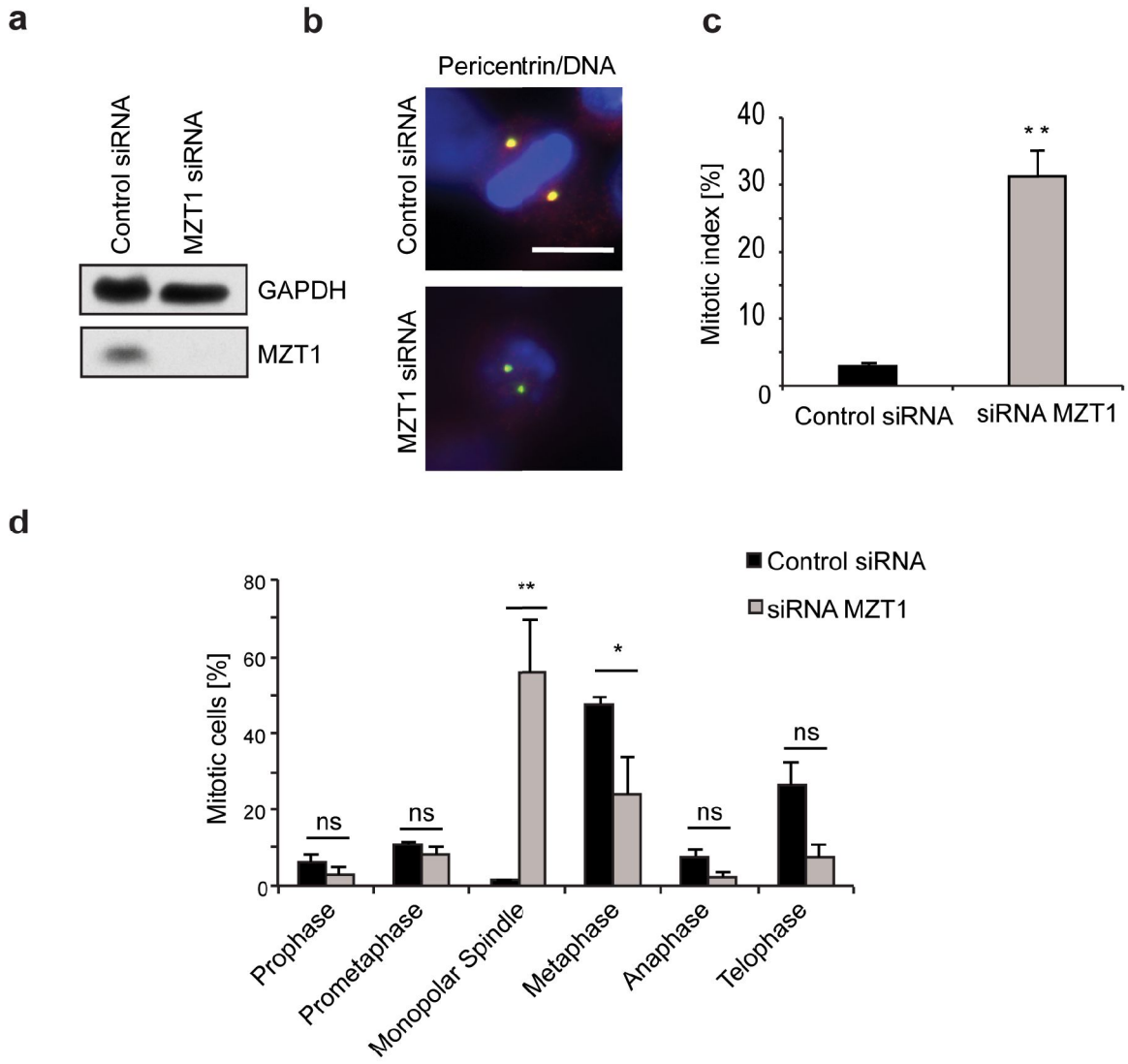

Figure 29. MZT1 is required for bipolar spindle assembly and mitotic progression.

(a) HeLa cells were treated with siRNA against MZT1 for 72 hours. Whole cell lysates were analyzed by Western blotting with MZT1 antibody to test the efficiency of the depletion. (b) Representative images of HeLa cells treated as in a, showing normal metaphase spindle in control cells and monopolar spindle in MZT1 depleted cells. Cells were fixed and stained with anti Pericentrin antibody (green), anti γ-tubulin antibody (red) and DNA was visualized with DAPI (blue) (Scale bar = 10 μM). (c) Quantification of the percentages of mitotic cells after treatment as in a (values are the means from n=3 independent experiments; at least 300 cells per condition; error bars: s.e.m.; ns, not significant; ***P<0.01 by one way ANOVA followed by Bonferroni's test) . (d) Histogram showing the distribution of mitotic cells in the different mitotic phases or with monopolar spindle. >100 mitotic cells analyzed per experiment, n3 experiments (error bars: s.e.m.; ns, not significant; *P<0.1; **P<0.01 by one way ANOVA followed by Bonferroni's test).

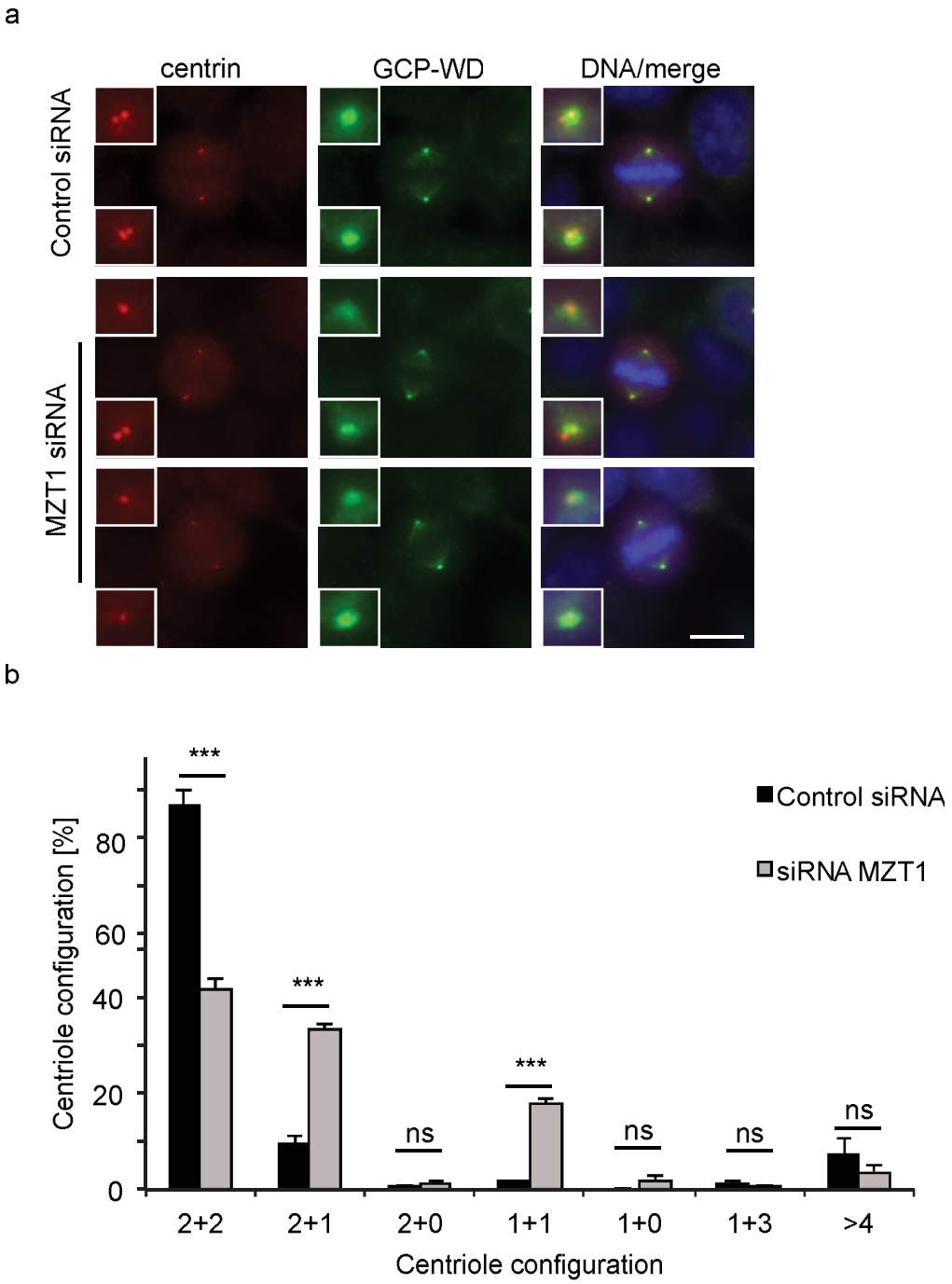

Figure 30. MZT1 is required for centriole duplication.

(a) Representative images of HeLa cells depleted of MZT1 stained with centrin (red) and GCP-WD (green) antibodies. DAPI was used for DNA (blue). Insets are magnifications of the centrosomes. (b) Mean percentages of cells with different centriole configurations in control and MZT1 depleted cells (>100 centrioles per condition; error bars s.e.m.; ns, not significant; ***P<0.001 by one way ANOVA followed by Bonferroni's test).

3.3. MZT1 is required for the targeting of γTuRC to the centrosome but not for γTuRC integrity

The phenotypes that I observed suggested that MZT1 was important for the function of γTuRC at centrosomes. To test if MZT1 has a role in the centrosomal localization of γTuRC, I quantified the fluorescence intensity of γ-tubulin at the centrosomes in cells depleted of MZT1 during interphase and mitosis. The depletion of MZT1 leads to a ~75% reduction in the amount of γ-tubulin at mitotic centrosomes and a ~60% reduction at interphase centrosomes (n=100 centrosomes from three independent experiments), while the intensity of the pericentrin signal was unaffected (Figure 31).

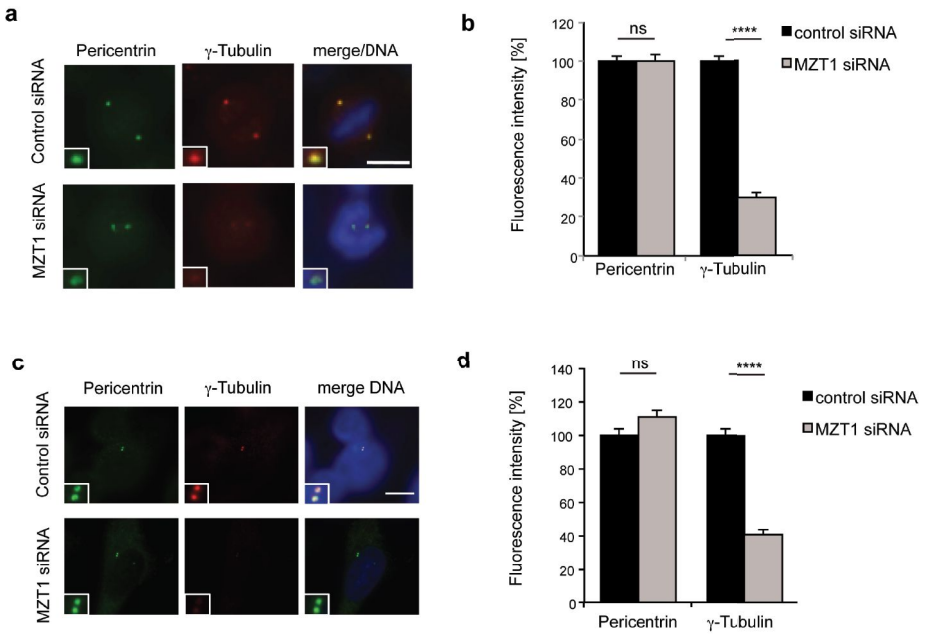

Figure 31. MZT1 is required for the targeting of γ-tubulin to centrosomes.

(a) Representative microscopy images of HeLa cells in mitosis treated for 72 hours with siRNA against MZT1, fixed and stained with γ-tubulin (red) and pericentrin (green) antibodies. DAPI were used to visualize DNA (blue). Insets are magnifications of the centrosomes (Scale bar = 10 μm). (b) Quantification of the fluorescence intensity of γ-tubulin and pericentrin staining at the centrosomes in cells treated as in a. The data is presented in percentages, control values were set to 100% (Error bars s.e.m., data from at least 100 centrosomes from experiment in three independent experiments). (c) Representative microscopy images of HeLa cells in interphase treated as in a. (d) Quantification of the fluorescence intensity of γ-tubulin and pericentrin staining at the centrosomes in interphase as indicated in b. (ns, not significant; **P<0.01; ****P<0.0001 by one way ANOVA followed by Bonferroni's test).

My next goal was to learn more about the mechanism by which MZT1 regulates γTuRC targeting to centrosomes. One possibility was that MZT1 was required to assemble the γTuRC (since only intact γTuRC can target to centrosomes) and a second possibility was that MZT1 was necessary for attaching γTuRC at centrosomes, directly or indirectly, by mediating interaction of γTuRC with other targeting factors. To test the first possibility I analyzed cell extract from HeLa cells treated with MZT1 siRNA by fractionation on a 10-40% sucrose gradient. The results indicate that the stability of the complex is not affected in cells depleted of MZT1 (Figure 32).

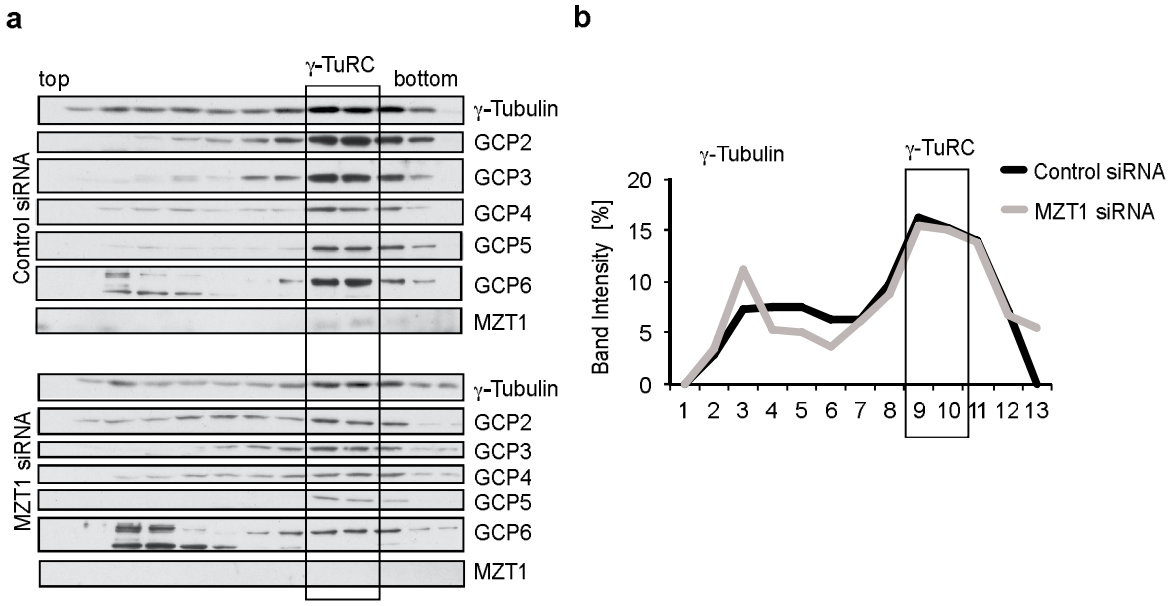

Figure 32. Depletion of MZT1 does not affect assembly or stability of the γTuRC.

(a) HeLa cell extracts treated for 72 hours with siRNA against MZT1 or luciferase as control were subjected to centrifugation through a 10-40% sucrose density gradient, 13 fractions were collected and analyzed by Western blot with antibodies for the γTuRC proteins, showing that endogenous MZT1 cofractionates with γ-TuRC. (b) Distribution of γ-tubulin band intensity in the Western blot of the sucrose gradient fractions indicating that MZT1 depletion does not impair γTuRC integrity.

3.4. MZT1 mediates the interaction of GCP-WD with the γTuRC

γ-Tubulin recruitment to centrosomes and mitotic spindles is dependent on the targeting factor GCP-WD (Haren et al., 2006; Lüders et al., 2006). To study the possibility that the reduction of γ-tubulin at the centrosomes is due to a mislocalization of GCP-WD I quantified the intensity of GCP-WD in cells depleted of MZT1. MZT1 depletion leads to a reduction of 28% in the amount of GCP-WD at centrosomes in mitotic arrested cells compared to control cells (Figure 33a, b). In contrast, cells in interphase present an ~18% increase in the GCP-WD signal compared to control cells (Figure 33c, d). I didn't explore the basis of these changes in the amount of centrosomal GCP-WD, but in any case they could not explain the much stronger reduction in centrosomal γ-tubulin after MZT1 depletion that I described above (Figure 33c and Figure 33d).

To test if GCP-WD can interact with the γTuRC in absence of MZT1 I immunoprecipitated endogenous GCP-WD from cells depleted of MZT1, and analyzed the samples by Western blot against GCP6 and γ-tubulin. I found that the interaction of GCP-WD with the γTuRC is lost upon the depletion of MZT1 (Figure 34). Altogether these results suggest that MZT1 may function as an adaptor mediates the interaction between the targeting factor GCP-WD and the γTuRC to allow recruitment of the complex to the centrosome.

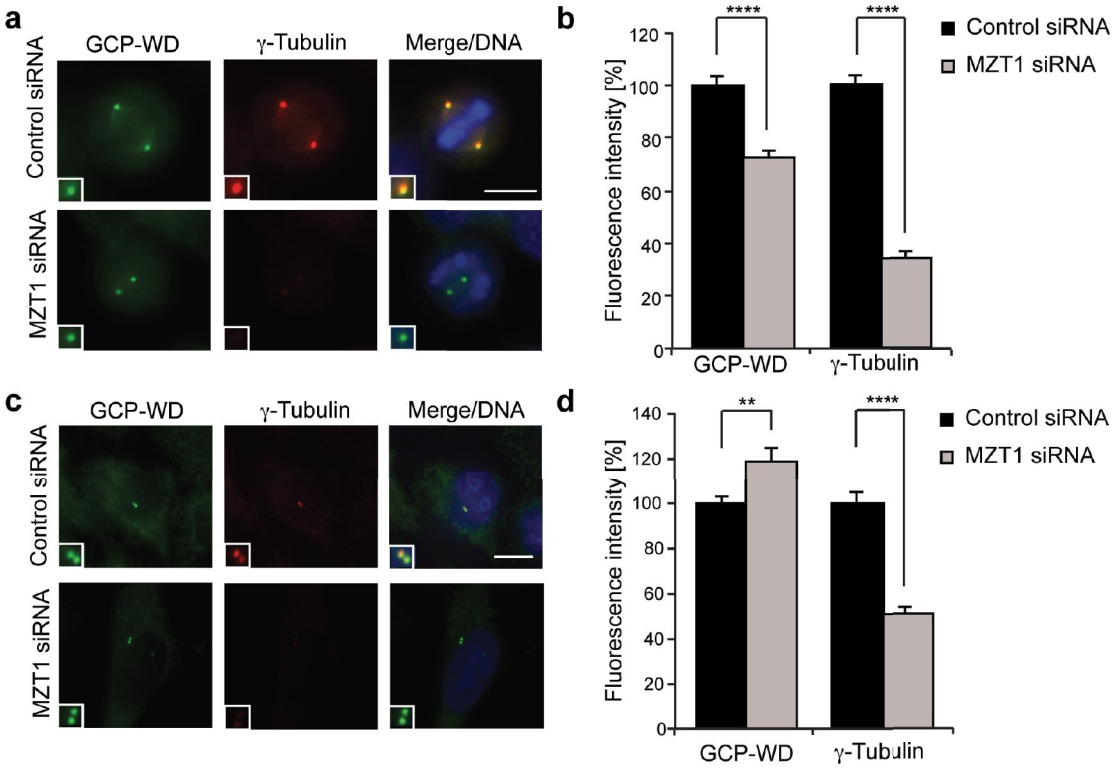

Figure 33. GCP-WD localization to centrosomes does not strictly dependent on MZT1.

(a) Microscopy images of mitotic HeLa cells depleted of MZT1, fixed and stained for γ-tubulin (red), GCP-WD (green) and DAPI for DNA (blue). Insets are magnifications of the centrosomes (Scale bar = 10 μm). (c) Representative images of cells in interphase, treated as in a. The fluorescence intensity for γ-tubulin and GCP-WD at the centrosome was quantified for cells in mitosis (b) and interphase (d), respectively. The mean values of at least 100 centrosomes from three independent experiments were blotted a percentage intensities in control cells, control values were set to 100% (error bars s.e.m., n=40 centrosomes, **P<0.01; ****P<0.0001 by one way ANOVA followed by Bonferroni's test).

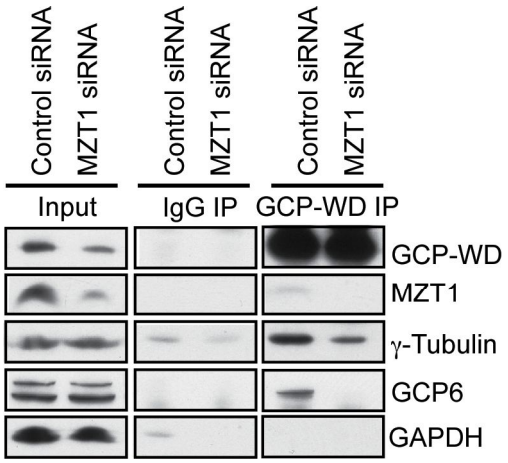

Figure 34. MZT1 mediates the interaction of GCP-WD with the γTuRC.

(a) Extracts of HeLa cells treated with siRNA against MZT1 were immunoprecipitated with GCP-WD or IgG control antibodies; the samples were analyzed by Western blotting and probed with antibodies against the indicated proteins.

3.5. MZT1 interacts with GCP2, GCP3, GCP5 and GCP6

3.5.1. MZT1 interacts with the N-terminal extension of GCP2, GCP3, GCP5 and GCP6

To further characterize the interaction of MZT1 with the γTuRC in mammalian cells I performed co-immunoprecipitation experiments from cells co-transfected with FLAG tagged N-terminal domain of each one of GCP2-GCP6 in combination with GFP-MZT1. The full-length proteins and the fragments analyzed are represented in the scheme in Figure 35a. By immunoprecipitation of the FLAG-tagged GCP N-terminal fragments I was able to detect an interaction of GFP-MZT1 with FLAG-tagged GCP3 1-552. In addition, I found that in mammalian cells MZT1 also interacts with the corresponding N-terminal fragments of GCP5 and GCP6, but not GCP4 The interaction of MZT1 with the N-terminal region of GCP3, GCP5 and GCP6 was lost by the expression of fragments that contain only the Grip domain but lack the region corresponding to the N-terminal extension (Figure 35c). These observations were confirmed by a yeast two

hybrid assay done in collaboration with Susana Eibes in the group of Joan Roig (IRB Barcelona). Moreover, the yeast two hybrid assay indicated a weak interaction of MZT1 with the N-terminal region of GCP2 (Figure 35b).

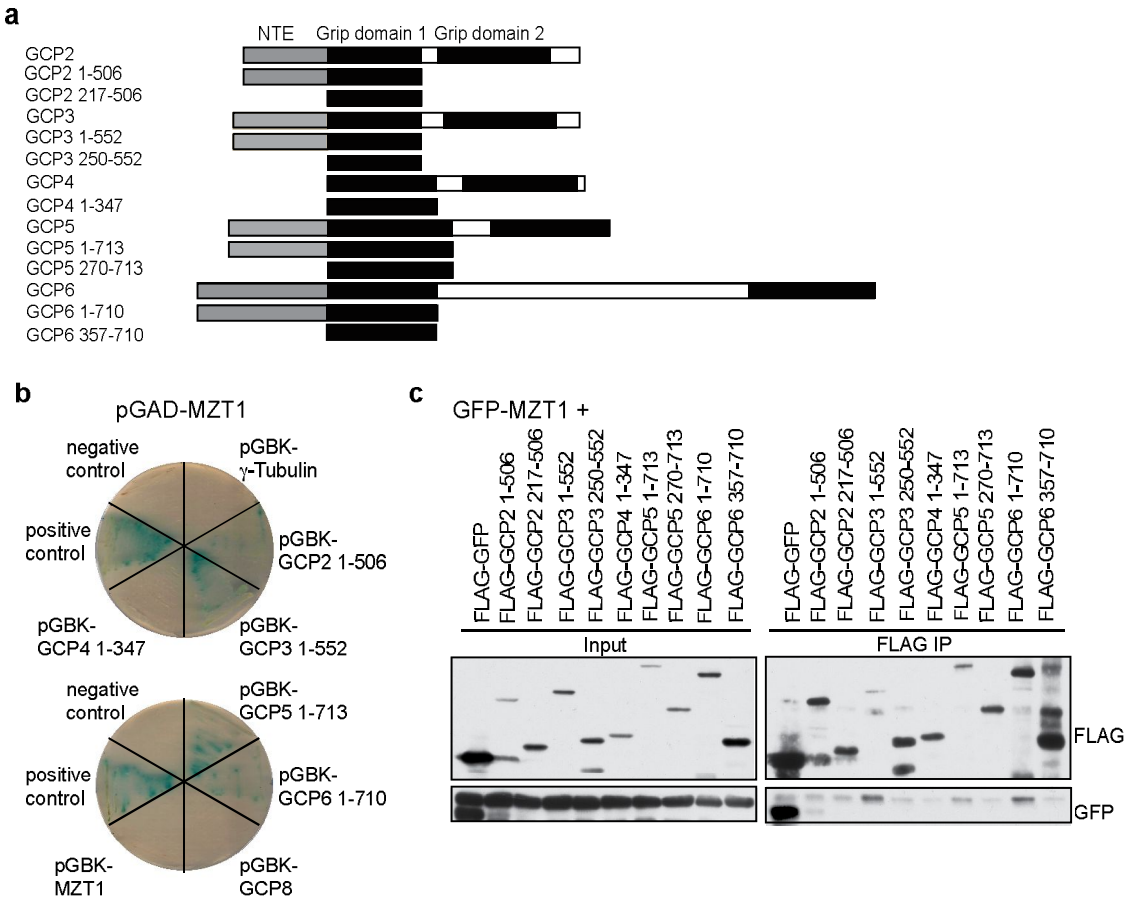

Figure 35. MZT1 interacts with the N-terminal extension of GCP2, GCP3, GCP5 and GCP6.

(a) Schematic representation of the GCPs domains. NTE: N-terminal extension, shaded in grey; Grip domains shaded in black. (b) Yeast two hybrid assay to test interaction of MZT1 with the N-terminal domains of the different GCPs. The N-terminal fragments GCP2 1-506, GCP3 1-552, GCP4 1-347, GCP5 1-713, GCP6 1-710 were fused to a Gal4 activation domain (pGADT7) and MZT1 full length to the Gal4 DNA binding domain (pGBKT7). The interactions were assessed by growth on medium lacking histidine and adenine and by positive α-Galactosidase activity (in collaboration with Susana Eibes, Joan Roig, IRB Barcelona). (c) GFP-MZT1 co-expressed with FLAG-tagged GCPs N-terminal constructs were transiently expressed in HEK293 cells, and immunoprecipitated with anti-FLAG antibody. The samples were analyzed by Western blotting and probed with antibodies for FLAG and GFP. GFP-MZT1 co-immunoprecipitation N-terminal extension of of GCP3, GCP5 and GCP6.

3.5.2. MZT1 interacts with a hydrophobic motif within the first amino acids of GCP3, GCP5 and GCP6

In order to identify a potential MZT1 binding motif in the N-termini of GCP3, GCP5, and GCP6, I performed multiple sequence alignments using Geneious software (6.1.2 version). This led to the identification of a short hydrophobic motif that is conserved in GCP2, GCP3, GCP5, and GCP6 (Figure 36a). Since the interaction between MZT1 and GCP2 was weak, I focused on the proteins that interacted stronger with MZT1. By PCR mutagenesis I generated the following mutants: GCP3 I93A/L94A/L97A, GCP5 I110A/L111A/L114A and GCP6 V110/L111A/L114A, termed GCP3 3A, GCP5 3A and GCP6 3A respectively. The interaction of GCP3 3A, GCP5 3A and GCP6 3A mutants with MZT1 was tested by the yeast two hybrid assay (Figure 36b). The interaction between MZT1 and the mutants was lost while the wild type controls were able to interact with MZT1 as I described above.

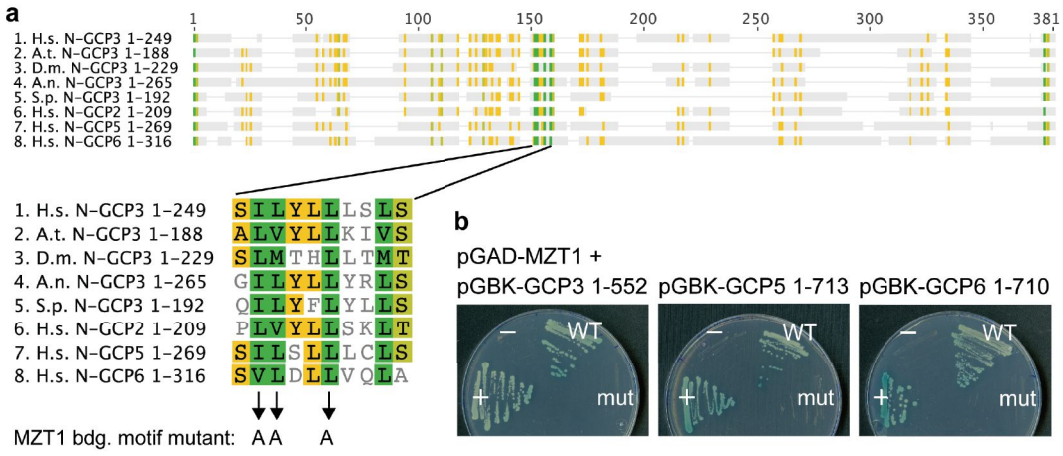

Figure 36. MZT1 interacts with a conserved hydrophobic motif within the N-terminal extension of GCP2, GCP3, GCP5 and GCP6

(a) Amino acid sequence alignment of the N-terminal extension of GCP2, GCP3, GCP5 and GCP6 in different species (*H.s., Homo sapiens; A. t., Arabidopsis thaliana; S. p., Schizosaccharomyces pombe; D. m., Drosophila melanogaster; A. n., Aspergillus nidulans*). The magnification indicates the conserved amino acids and the point mutations for GCP3, GCP5 and GCP6. (b) Yeast two hybrid for N-terminal wild type or 3A mutants for GCP3 I93A/L93A/L97A, FLAG-GCP5 I110A/L111A/L113A.

3.5.2.1. MZT1 binding mutants can interact with the γTuRC proteins but not with GCP-WD

Next I tested whether mutation of the MZT1 binding motif affected the function of the full-length GCPs. By immunoprecipitacion of full-length FLAG-tagged wild type GCPs or 3A mutants I confirmed that the mutants were defective in binding to endogenous MZT1 (Figure 37). Importantly, the mutants still interacted with other γTuRC proteins such as GCP2, GCP4 and GCP5, suggesting that the mutants can integrate into the complex, but cannot interact with MZT1 (Figure 37). As expected, these mutants also failed to coprecipitate GCP-WD, in agreement with my previous observation that MZT1 was required for GCP-WD to interact with the γTuRC (Figure 34).

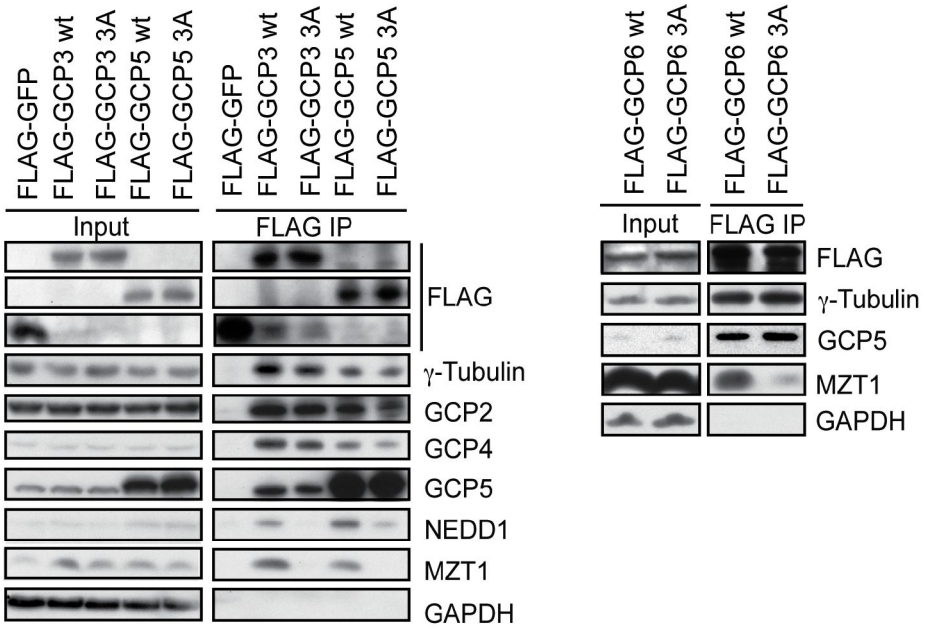

Figure 37. The GCP MZT1 binding motif mutants interact with other γTuRC components but are not able to interact with MZT1 or GCP-WD.

Cell extract of HEK293 cells, transiently expressing FLAG-GFP, FLAG-GCP3 wt, FLAG-GCP3 3A, FLAG-GCP5 wt, FLAG-GCP5 3A, FLAG-GCP6 wt and FLAG-GCP6 3A were immunoprecipitated with anti-FLAG antibody and analyzed by Western blotting probed with antibodies for the indicated proteins.

I then asked whether the MZT1 binding mutants will also be affected in the targeting to the centrosome, as is the case for γ–tubulin in the absence of MZT1. To determine if the mutants can be targeted to the centrosomes I quantified the centrosomal fluorescence intensity in U2OS cells expressing the FLAG tagged wild type proteins or 3A mutants after staining with anti-FLAG antibodies. By this analysis I could show that the 3A mutants did not localize efficiently to the centrosomes when compared to the wild type proteins (Figure 38). The fluorescence intensity of the FLAG signal at the centrosomes was reduced by 56%, 44%, and 58% in GCP3 3A, GCP5 3A, and GCP6 3A expressing cells, respectively, compared to the signals in cells expressing the wild type proteins (Figure 38b).

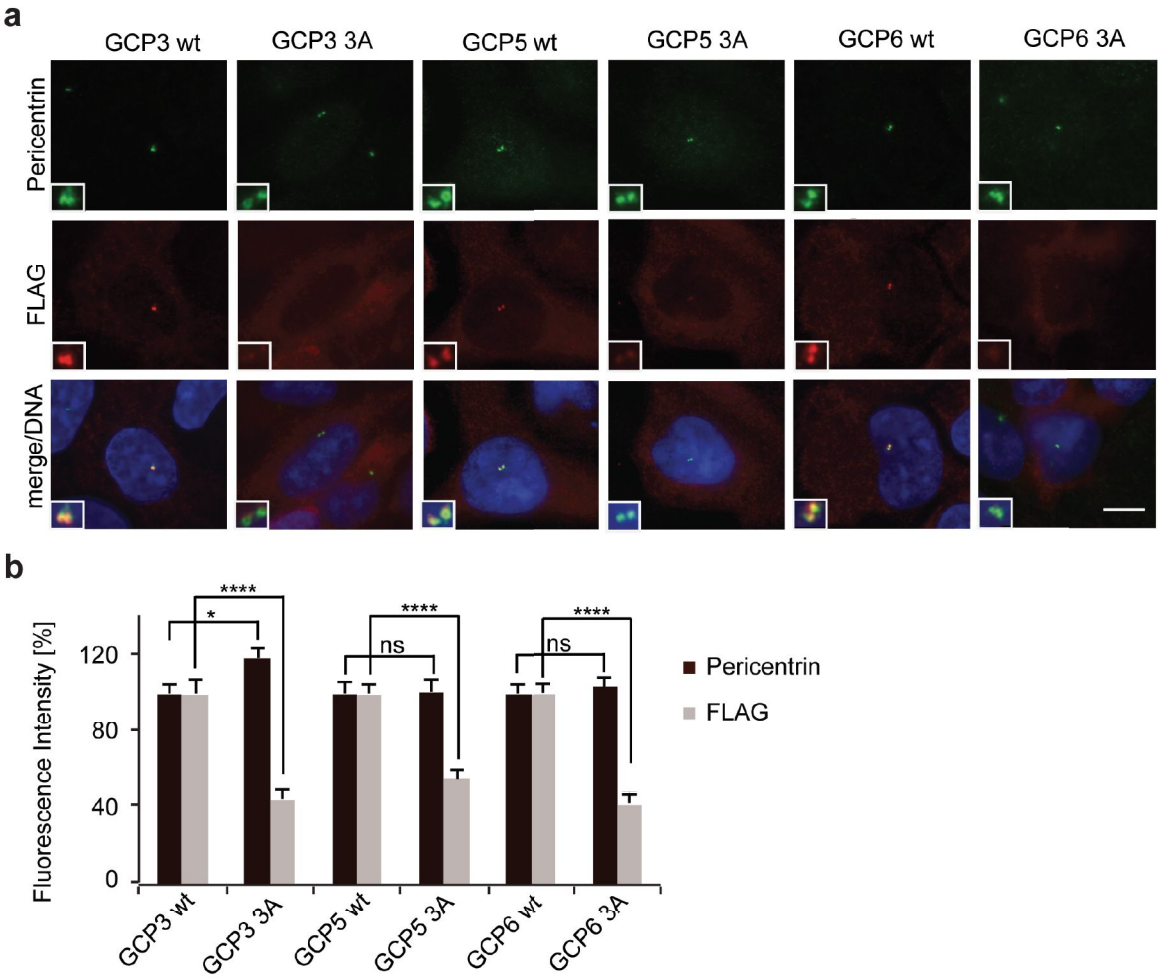

Figure 38. The MZT1 binding motif is required to recruit GCPs to centrosomes

(a) U2OS cells transiently expressing FLAG-GCP3 wt, FLAG-GCP3 3A, FLAG-GCP5 wt, FLAG-GCP5 3A, FLAG-GCP6 wt and FLAG-GCP6 3A, fixed after 48 hours and stained with antibodies for against FLAG (red) and pericentrin (green). DAPI was used to stain DNA (blue). Insets are magnifications of the centrosomes (Scale bar = 10 μm). (b) Quantification of fluorescence intensity of FLAG staining at centrosomes, showing an impaired targeting of GCP mutants to centrosomes. The mean values from three independent experiments were plotted as percentage intensities relative to control cells. Control values were set to 100% (error bars s.e.m., at least 60 centrosomes from 3 different experiments, ns, not significant; *P<0.1; ****P<0.0001 by one way ANOVA followed by Bonferroni's test).

3.6. MZT1 favors microtubule nucleation

3.6.1 MZT1 over-expression induces cytoplasmic nucleation

It was previously shown that the over-expression of the γTuRC-binding domain CDK5RAP2 51-100 (CM1 domain) in U2OS cells induces an eightfold increase in the γTuRC-dependent cytoplasmic nucleation activity compared to control cells (Choi et al., 2010). To test the possible involvement of MZT1 in this process I performed a microtubule nucleation assay in U2OS cells over-expressing GFP-MZT1 and compared with cells expressing GFP or GFP-CDK5RAP2 CM1. GFP-MZT1 over-expression increased microtubule nucleation twofold compared to GFP expressing control cells, while GFP-CDK5RAP2 CM1 increased the nucleation activity fivefold (Figure 39).

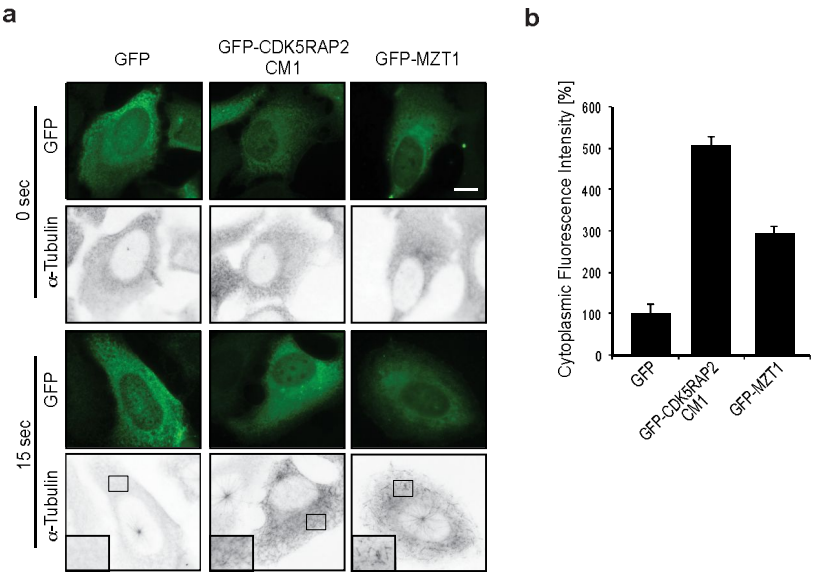

Figure 39. MZT1 promotes γTuRC-dependent cytoplasmic nucleation

(a) U2OS transiently expressing GFP or GFP-MZT1 were subjected to microtubule nucleation. Microtubules were depolymerized on ice for 30 minutes (time point 0) and warmed up at 37 °C to allow microtubule regrowth (time point 15 sec). Cells were fixed and stained with antibodies against GFP (green) and α-tubulin (inverted image). Insets are magnifications of cytoplasmic microtubules (Scale bar = 10 μm). (b) The intensities of the cytoplasmic microtubules were quantified by measuring fluorescence intensity of α-tubulin from the complete area of the cells, and subtracting the centrosome microtubule aster (Error bars s.e.m., data of 80 cells from three different experiments).

3.6.2. Microtubule nucleation activation by CDK5RAP2 CM1 is dependent on γTuRC components

Microtubule nucleation activation induced by CDK5RAP2 CM1 requires an intact γTuRC, as it was shown that cells depleted of the structural component GCP4 and overexpressing GFP-CDK5RAP2 CM1 fail to nucleate microtubules (Choi et al., 2010). My previous results suggested that MZT1 is not required for γTuRC integrity per se, but may stabilize the complex and/or alter its properties, and stimulate its nucleation activity either directly or indirectly. I asked whether MZT1 was required for CDK5RAP2 CM1 to stimulate γTuRC nucleation activity in analogy to MZT1 being required for GCP-WD/NEDD1 to interact with and target γTuRC to centrosomes.

To test this I performed a microtubule regrowth experiment with U2OS cells over-expressing GFP-CDK5RAP2 CM1 or GFP and treated with control or MZT1 siRNA. As a control I expressed GFP-CDK5RAP2 CM1 in cells treated with control or GCP2 siRNA, to disrupt γTuRC. I collected samples at 10 sec after allowing microtubule regrowth and fixed with cold methanol. The cells were stained for GFP and α-tubulin (Figure 40a). After image capturing I quantified the cytoplasmic α-tubulin fluorescence. Cells depleted of GCP2 and expressing GFP displayed a decrease of α-tubulin intensity at the cytoplasm of about 0.5 compared to control. While cells treated with control siRNA and transfected with GFP-CDK5RAP2 CM1 displayed a fivefold increase α-tubulin intensity compared to control, but the over-expression of GFP-CDK54AP2 CM1 in GCP2 and MZT1 depleted cells only displayed 1.5fold increase in α-tubulin intensity values (Figure 40b). These observations suggest that the intact γTuRC is required to increase the microtubule nucleation induced by CDK5RAP2 CM1 expression and that MZT1 depletion abolishes stimulation of microtubule nucleation in a comparable way as γTuRC disruption by GCP2 depletion.

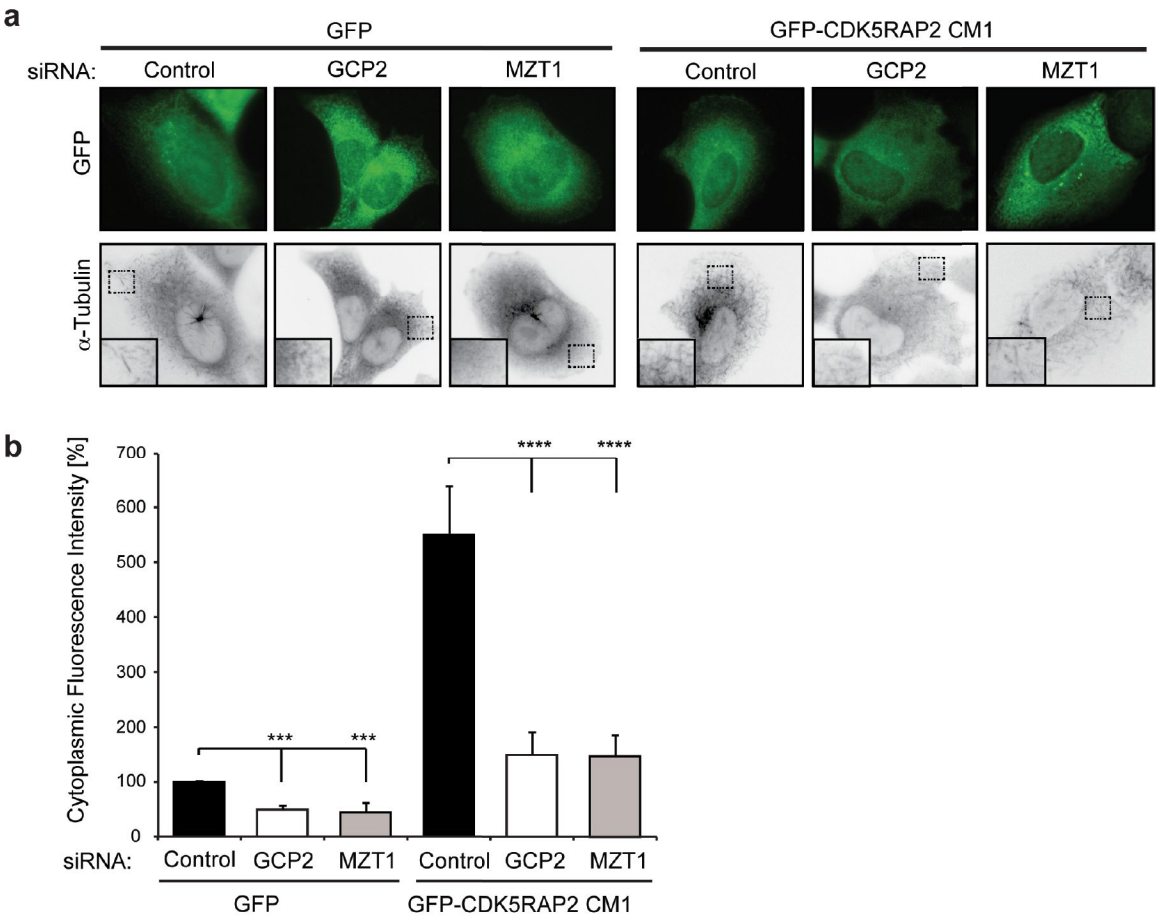

Figure 40. Activation of microtubule nucleation by CDK5RAP2 CM1 requires MZT1

(a) U2OS cells transfected with siRNA against Luciferase, GCP2 or MZT1, followed by GFP or GFP-CDK5RAP2 CM1 transfection. After microtubule regrowth (time point 10 sec) cells were fixed and stained with antibodies against GFP (green) and α-tubulin (inverted image). Insets are magnifications of cytoplasmic microtubules (Scale bar = 10 μm). (b) Quantification of cytoplasmic fluorescence intensity of α-tubulin from cells in treated as in a . The mean values of at least 80 cells from three independent experiments were blotted a percentage intensities in control cells, control values were set to 100% (error bars s.e.m.,***P<0.001; ****P<0.0001 by one way ANOVA followed by Bonferroni's test).

Since my previous results demonstrated that MZT1 is required for GCP-WD to interact with the γTuRC, I wondered whether if MZT1 was also necessary for CDK-RAP2 interaction with the γTuRC. This possibility was addressed, in collaboration with Artur Ezquerra from my group, by expressing GFP or GFP-CDK5RAP2 CM1 in cells Hek293 cells treated with Control siRNA or siRNA against MZT1, following the immunoprecipitation using an antibody anti GFP. The interaction of the CM1 domain with the γTuRC was strongly affected upon the depletion of MZT1, compared to control. Thus we conclude that MZT1 mediates the interaction of CDK5RAP2 with the γTuRC.

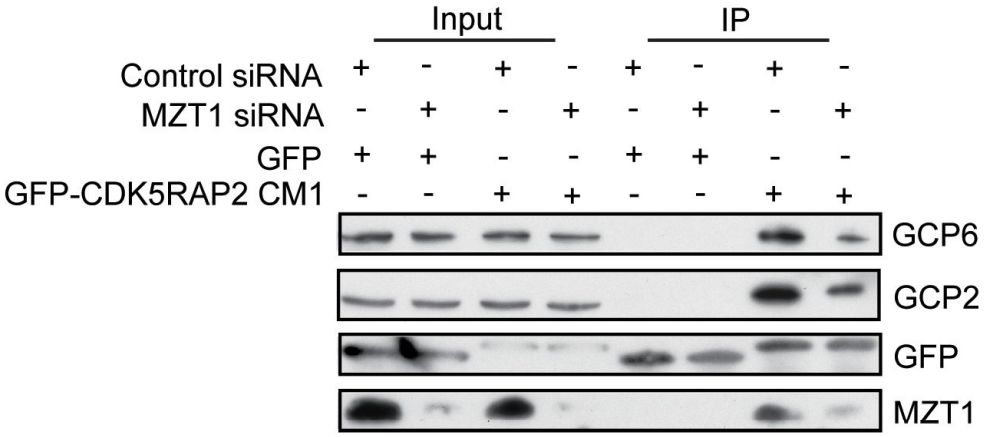

Fig. 41. MZT1 mediates the interaction of CDK5RAP2 with the γTuRC.

Extracts of Hek293 cells treated with siRNA against MZT1 and expressing GFP or GFP-CDK5RAP2 CM1 were immunoprecipitated with GFP antibodies. The samples were analyzed by Western blotting and probed with antibodies against the indicated proteins. (Artur Ezquerra/Lüders group).

Discussion

4.1. MZT1 is a core member of the γTuRC essential for mitotic progression

4.1.1. MZT1 interacts with core γTuRC subunits and co-localizes with γ-tubulin throughout the cell cycle

MZT1 is a highly conserved protein, described as a member of the γTuRC in mammalian cells, *Arabidopsis thaliana* and *Schizosaccharomyces pombe* (Dhani et al., 2013; Hutchins et al., 2010; Janski et al., 2012; Masuda and Toda, 2016b; Masuda et al., 2013; Teixidó-Travessa et al., 2010) and required for targeting γ-tubulin complexes to MTOCs. Despite this information its molecular function remained largely unknown. In part, a more detailed analysis was hindered by the lack of antibodies that allow detection of endogenous MZT1 (Batzenschlager et al., 2013; Dhani et al., 2013; Hutchins et al., 2010).

In my thesis I have addressed this issue by first generating suitable antibodies directed against human MZT1 and by developing a protocol that allows detection of endogenous MZT1 by western blotting.

In agreement with previous observations, I identified all Grip-GCPs and γ-tubulin to interact with MZT1 by immunoprecipitation of endogenous MZT1 from HeLa cells extracts (Figure 27a). Moreover, I found that the set of MZT1 interacting proteins includes the targeting factor GCP-WD, contrary to what was described in plants. Here the pull-down of GFP tagged GIP1a/MZT1 coprecipitated all γTuRC proteins except GCP-WD (Nakamura et al., 2012). This led the authors to suggest that in plants MZT1 is a member of a subpopulation of γTuC, different from the population that contains GCP-WD (Nakamura et al., 2012). It remains to be elucidated whether the conflicting result is due to evolutionary divergences between plants and animals, or due to differences in the methods that were used. However, my study is the first to analyze endogenous untagged MZT1. Thus, I conclude that MZT1 and GCP-WD can be part of the same γTuRCs, at least in human cells.

MZT1 was shown to co-localize with γ-tubulin throughout the cell cycle (Dhani et al., 2013; Hutchins et al., 2010; Janski et al., 2012; Masuda et al., 2013; Nakamura et al., 2012). In interphase fission yeast Mzt1/Tam4 localizes to the SPB and to the MT bundles. During mitosis MZT1 localizes to the SPBs at the spindle poles, and during anaphase/telophase to the equatorial region known as eMTOC (Dhani et al., 2013; Masuda et al., 2013).

The plant MZT1 homologs tagged with GFP localize to the nuclear envelope and to cortical MT, and during mitosis localize to spindle microtubules (Janski et al., 2012; Nakamura et al., 2012). Moreover GIP1a and GIP1b were shown to localize to active nucleation sites in the interphase cortical microtubule array(Nakamura et al., 2012).

To better characterize the distribution of MZT1 in human cells, I analyzed the localization of MZT1 at different stages of the cell cycle by immunofluorescence microscopy of cells expressing GFP-MZT1. I found that MZT1 co-localized with γ-tubulin throughout the cell cycle, including centrosomes, spindle MTs, and the MTs at the midbody region during cytokinesis (Figure 27). The localization of MZT1 to the midbody MTs in human cells was not described before. Together my results suggest that MZT1 is a core component of the human γTuRC present at all γTuRC targeting sites.

4.1.2. MZT1 is necessary not only for proper spindle assembly but also centriole duplication

The depletion of MZT1 in human cells causes severe mitotic spindle defects. Depleted cells lack centrosomal γ-tubulin and arrest in mitosis with a monopolar spindle configuration (Hutchins et al., 2010). Similarly, double deletion mutants of the plant MZT1 orthologs GIP1/GIP1a and GIP2/GIP1b are embryonic lethal due to abnormal spindle microtubule distribution and chromosome mis-segregation (Janski et al., 2012; Nakamura et al., 2012). Additionally, the double mutants exhibit alterations of the nuclear membrane associated with abnormal distribution

of the nuclear pore complexes (Batzenschlager et al., 2013). In yeast Mzt1/Tam4 is essential for cell growth and for mitotic and interphase MT organization, and plays a role in cytokinesis (Dhani et al., 2013; Masuda et al., 2013). In this work I could confirm that in human cells MZT1 is indeed required for the targeting (Figure 31) of the γTuRC to centrosomes and for proper spindle formation (Figure 29).

Since MZT1 depletion leads to an accumulation of cells with monopolar spindle configuration, to explore if this may also be related to centriole duplication defects, I quantified the centriole number in MZT1-depleted mitotic cells. My results indicate that MZT1 is indeed necessary for centriole duplication. In agreement with my observations, γTuRC proteins have been implicated in centriole duplication previously (Bahtz et al., 2012; Haren et al., 2006; Raynaud-Messina et al., 2004; Ruiz et al., 1999; Shang et al., 2002).

γ-Tubulin is necessary for basal body duplication in *Paramecium* and *Tetrahymena thermophila,* (Ruiz et al., 1999; Shang et al., 2002). In human cells, overexpression of Polo-like kinase 4 (Plk4) induces centrosome amplification and γ-tubulin is required for this process (Kleylein-Sohn et al., 2007). Upon the depletion of γ-tubulin in *Drosophila* S2 cells most of the centrioles were shortened, suggesting a role of γ-tubulin in centriole morphogenesis (Raynaud-Messina et al., 2004). Human GCP4, GCP5 and GCP6 were also implicated in centriole duplication, their depletion results in a reduction of centriole number, with the strongest defect caused by GCP6 depletion (Bahtz et al., 2012; Neus Teixido/Lüders group, unpublished). GCP-WD is also required for centriole duplication by targeting of γTuRC to the centrosomes (Haren et al., 2006; Lüders et al., 2006). Therefore, one can assume that similar to GCP-WD, the role of MZT1 in centriole duplication is based on its function in targeting γTuRC to centrosomes.

4.2. Regulation of the γTuRC by MZT1

4.2.1. MZT1 promotes the fractionation of γTuRC as high molecular weight complex

I have shown by sucrose gradient fractionation of HeLa cell extracts over-expressing GFP-MZT1, that GFP-MZT1 distributes broadly along the gradient, including the fractions corresponding to the γTuRC, similar to the distribution of MZT1-LAP expressed in mammalian cells (Hutchins et al., 2010). Interestingly I additionally found that MZT1 over-expression promotes an enrichment of the GCPs in fractions corresponding to the γTuRC, and a slight shift to higher molecular weight fractions, suggesting that MZT1 induces a stabilization of the γTuRC (Figure 28).

This observation and the fact that endogenous MZT1 is present mainly in the sucrose gradient fractions corresponding to the γTuRC but not smaller fractions (Figure 32), indicate that MZT1 binds preferably to the γTuRC over the γTuSC in mammalian cells. Supporting this idea, MZT1-LAP tagged immunoprecipitates γ-tubulin from fractions corresponding to the γTuRC, but not from γTuSC fractions (Hutchins et al., 2010).

Interestingly, recent findings support the idea that MZT1 cooperates with the γTuRC to control the targeting and MT nucleation activity. Even though fission yeast GCP4/Gfh1, GCP5/Mod21 and GCP6/Alp16 are non-essential for viability, the deletion of GCP6/Alp16 together with expression of a temperature sensitive mutant MZT1 are lethal (Masuda and Toda, 2016). Surprisingly however, MZT1 is also present in the yeast *Candida albicans*, which, similar to *Saccharomyces cerevisiae*, contains only the γTuSC subunits γ-tubulin, GCP2, and GCP3. Thus one could speculate that MZT1's function may be not be linked to γTuRC per se, but rather to oligomeric γ-tubulin complexes, independent of their GCP composition.

4.2.2. MZT1 interacts with the N-terminal extension of GCP2, GCP3, GCP5 and GCP6

While in plants and in fission yeast MZT1 interacts with the N-terminal region of GCP3 (Dhani et al., 2013; Janski et al., 2012; Nakamura et al., 2012), I found that in mammalian cells MZT1 can also interact with the N-terminal fragments GCP2 1-506, GCP3 1-552, GCP5 1-713 and GCP6 1-710 (Figure 35). The interaction was demonstrated by yeast two hybrid assay and by co-immunoprecipitation of cell extract from cells expressing FLAG-tagged GCP fragments and GFP-MZT1. However, only a weak interaction with GCP2 was detected by yeast two hybrid, and the interaction was not seen by immunoprecipitation, suggesting that MZT1 interaction with GCP2 is transient or weak compared to GCP3, GCP5 and GCP6.

Despite these findings I obtained evidence that MZT1 interacts with a conserved hydrophobic motif present within the N-termini of all GCPs but GCP4 (Figure 35). Alanine mutation of hydrophobic residues in this motif abolished interaction with MZT1 by both yeast two hybrid and immunoprecipitation assay. Surprisingly two previous studies identified only the interaction of MZT1 with GCP3 (Dhani et al., 2013; Nakamura et al., 2012). However, in the study by Nakamura et al. (2012) the yeast two hybrid assay also showed weak interaction with GCP2 as well, even though the authors did not interpret this as a positive interaction. I can only speculate about the reasons for this discrepancy, but one possibility is that the authors in previous studies tested full length GCPs, which may not be expressed/folded properly in yeast and thus not allow detection in all cases. Another possibility is that there are species-specific differences in the strength of the binding when testing binary interactions.

According to the current model, γTuRC is assembled by lateral interactions of the N-terminal domains of the GCPs, and the interaction with γ-tubulin molecules with their C-terminal domain (Kollman et al., 2011; Teixidó-Travesa et al., 2012). Thus, in human cells the preferential binding of MZT1 to the γTuRC over the γTuSC might be favored by the presence of additional GCP subunits in the γTuRC. Therefore, several scenarios are possible for the binding of MZT1 to the

γTuRC. One possibility is that MZT1 binds to the N-terminal domain of GCP3, GCP5 and GCP6 at the seam of the γTuRC (Figure 42a), or can bind to all GCP3, GCP5 and GCP6 subunits that integrates the γTuRC, decorating the outer face of the complex (Figure 42b) or at the center of the complex (Figure 42c). The current model in the budding yeast, suggests that MZT1/Tam4 assembles into a hexamer oligomer that locates at the center of the complex by interacting with GCP3 (Dhani et al., 2012; Masuda et al., 2016). These observations are based on the fact that *in vitro* expressed and purified Mzt1-His can assemble into a tetramer, hexamer and dodecamer complexes (Dhani et al., 2013), but in mammalian cells the stoichiometry of MZT1 in the complex remains unknown. Thus, MZT1 can be associated to the complex as a tetramer at the seam of the complex, or even as a dodecamer around the complex. Thus, further biochemical studies are required to determine which are the oligomerization sites and which are the binding sites to the GCPs in the molecule of MZT1. Also, to determine the exactly position of MZT1 in the complex it will be necessary to do high-resolution structural analysis in purified complexes.

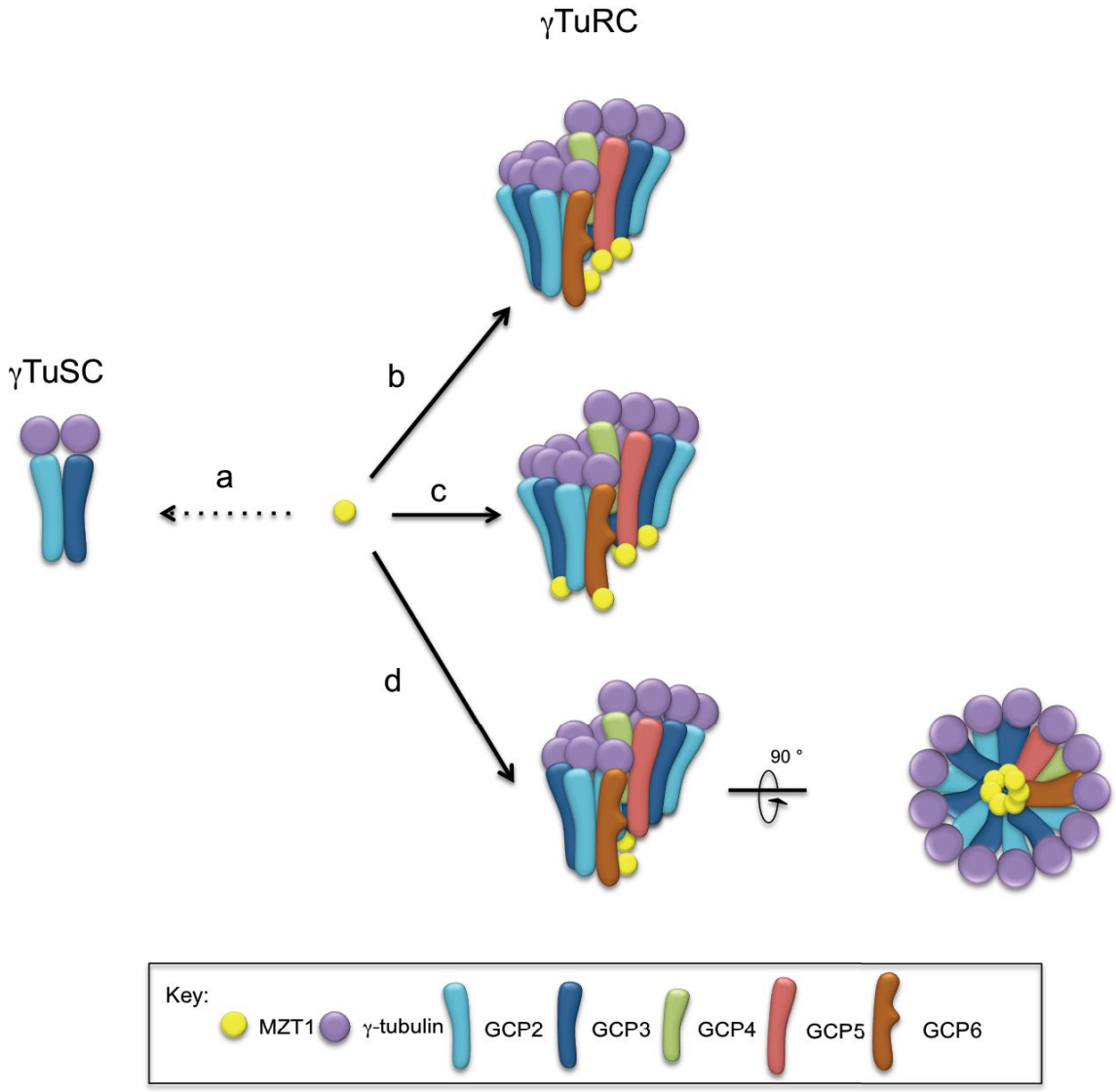

Figure 42. A model of MZT1 binding to the γTuRC.

Model of MZT1 interaction GCPs based on the speculative model of γTuRC assembly. (a) MZT1 does not efficiently interact with the γTuSCs. (b) MZT1 binds to the N-terminal of GCP3, GCP5 and GCP6 at the seam of the. γTuRC. (c) MZT1 can bind to every single GCP3 5 and 6 that integrates the complex. (d) MZT1 can interact with GCP3, 5 and 6 at the core of the complex. (Dot arrow indicates non/weak interaction, and continuous arrow indicates stable interaction).

4.2.3. MZT1 is required for the targeting of γTuRC to the centrosome mediated by the GCP-WD

Altogether my results and previous reports indicate that MZT1 mediates the targeting of the γTuRC to MTOCs, but the molecular mechanism that controls this process remained unknown. Here I provide evidences that MZT1 regulates the targeting of the γTuRC to centromes by promoting the interaction between GCP-WD and γTuRC.

The sucrose gradient fractionation of HeLa cell extracts treated with MZT1 siRNA indicated that γTuRC is assembled in absence of MZT1 (Figure 32). Moreover, GCP3 3A, GCP5 3A and GCP6 3A mutants lose the interaction with MZT1 (Figure 36, 37), but can still efficiently pull-down the other members of the γTuRC (Figure 37). Thus, the human MZT1 binding motif is not necessary for the assembly of GCPs into γTuRC. Rather, its is possible that MZT1 requires the preformation of the γTuRC in order to interact with the complex. Although, I didn't explore this possibility, it was shown that MZT1 targeting to the SPBs is dependent on the γTuSC integrity (Masuda et al., 2013). The loss of expression of fission yeast Mzt1/Tam4 does not affect γTuSC formation by GCP3/Alp6, GCP2/Alp4 and γ-tubulin/Gtb1 (Dhani et al., 2013).

Similarly, GCP-WD is also not necessary for the assembly of the γ-TuRC, but is required for the targeting to the MTOCs (Haren et al., 2006; Lüders et al., 2006). However, while GCP-WD can localize to centrosomes indepedently of γTuRC, Mzt1/Tam4 targeting to the SPBs is dependent on an intact γTuC (Masuda et al., 2013). I found that MZT1 is necessary for the interaction of the γTuRC with the targeting factor GCP-WD/NEDD1 in human cells. Immunoprecipitation of GCP-WD from cells lacking MZT1 revealed impaired interaction with the γTuRC (Figure 34). Accordingly, the GCPs 3A mutants failed to interact with GCP-WD (Figure 37), resulting in an impaired localization to centrosomes when compared to the wild type proteins (Figure 38). Since I found that MZT1 is not required for the stability of the complex per se, one possibility is that MZT1 induces a

conformational change in the γTuRC, favoring the interaction of GCP-WD with the complex, or that it links γTuRC to GCP-WD by direct binding to both components.

4.2.4. MZT1 promotes microtubule nucleation

In plants MZT1 localizes to active nucleation sites at pre-existing MTs at the cortical sites, suggesting that MZT1 is a component of active γTuRC (Nakamura et al., 2012). Furthermore, over-expression of MZT1 in fission yeast promotes nucleation of spindle MTs (Masuda and Toda, 2016b). My results indicate that MZT1 over-expression induces cytoplasmic MT nucleation, but not as strong as the induced by CDK5RAP2 CM1 (Figure 39). Thus I speculate, that MZT1 is not an activating factors by itself, but promotes γTuRC activation by other cellular factors.

γTuRC MT nucleation activity is further enhanced by the interaction with regulatory proteins, such as NME7 and CDK5RAP2 (Choi et al., 2010; Liu et al., 2014; Teixidó-Travesa et al., 2012). While the kinase NME7 induces a moderate activation of MT nucleation (Liu et al., 2014), CDK5RAP2 overexpression induces strong ectopic MT nucleation by binding to γTuRC through the CM1 motif. The over expression of the CM1 motif alone is sufficient to promote γTuRC MT nucleation activity in *vitro* and in cells (Choi et al., 2010). Taking advantage of this tool, I performed MT regrowth assays in cells over-expressing CDK5RAP2 CM1 motif and depleted of MZT1 or GCP2. I found that CM1-dependent activation of γTuRC nucleation activity is strongly impaired by the depletion MZT1 similar to the depletion of GCP2, a core component of the γTuRC (Figure 40). Furthermore, MZT1 mediates the interaction of the CM1 domain with γTuRC in a similar way as it mediates the interaction of GCP-WD/NEDD1 with the complex (figure 41). These results suggest that MZT1 generally mediates the binding of other targeting and activating proteins to the γTuRC. Thus I propose that MZT1 is

a key factor in the spatial control of MT nucleation at centrosomal MTOCs and non-centrosomal MTOCs.

All together my results suggests that MZT1 is major γTuRC regulator. It remains to determine if my observations in human cells are conserved among the different organisms that express MZT1. It will be interesting to determine if MZT1 is necessary for the MT nucleation induced by the Mto1/Mto2 in fission yeast, since Mto1 also contains a CM1 domain (Lynch et al., 2014). The lack of CM1-containing proteins in plants (Lin et al., 2014) raises the question whether MZT1 in plants is sufficient to induce MT nucleation by itself or whether it requires the association of other factors in analogy to my results in human cells.

Although it is widely accepted that γ-TuRC-associated proteins regulate MT nucleation it remains unclear how the nucleation is regulated (Kollman et al., 2011; Teixidó-Travesa et al., 2012). The best insights come from the budding yeast γTuSC, where a structural conformational change is necessary to promote the γTuSC oligomer activity (Kollman et al., 2010, 2011, 2015). To demonstrate that this configuration changes takes place in the γTuRC it is necessary to reconstitute the γTuRC *in vitro*, but the limitations to express *in vitro* the GCPs constitute a big challenge.

In summary I propose that in mammalian cells MZT1 binds to a preassembled inactive-γTuRC through the interaction with the conserved motif present in the extended N-termini of GCP3, GCP5 and GCP6 (probably including GCP2), allowing the recognition of fully assembled γTuRC (Figure 43). The binding of MZT1 "primes" the γTuRC for interaction with the adapter GCP-WD/NEDD1 for targeting γTuRC to centrosomes and/or CDK5RAP2 to activate the nucleation activity. One possibility is that MZT1 binding to the GCPs induces a conformational change at the N-terminal region of the GCPs exposing specific binding sites for the interaction with GCP-WD/NEDD1 and CDK5RAP2. A second possibility is that MZT1 generates a bridge between the targeting and activators

proteins and the γTuRC. Thus, by enabling specific recognition of γTuRC by targeting and activation factors, MZT1 spatially controls microtubule nucleation.

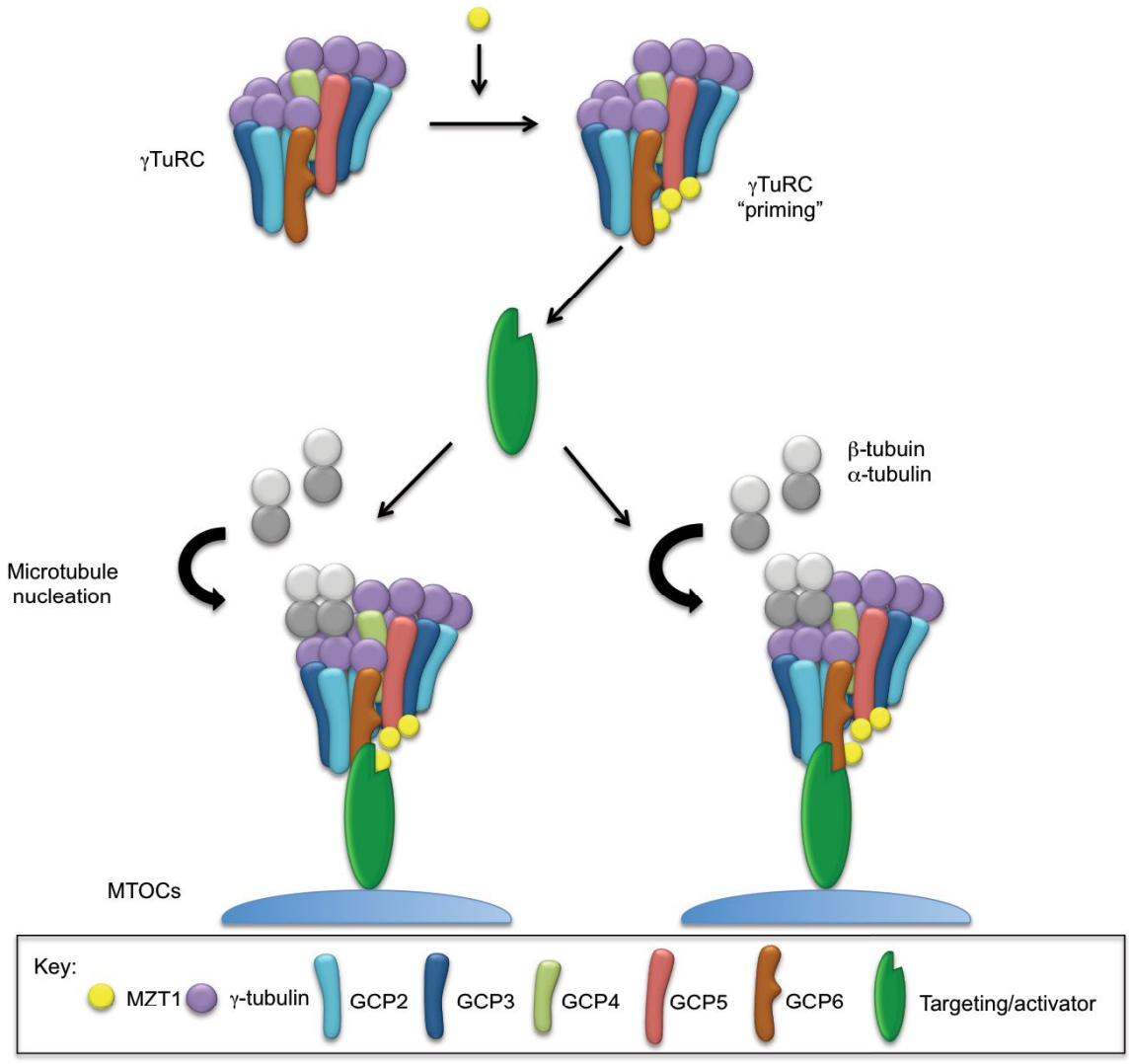

Figure 41. Model for γTuRC regulation by MZT1

The γTuRC assembled in absence of MZT1 is in an inactive configuration. The interaction of MZT1 pre-activates the complex ("priming"), which allows the complex to be recognized by targeting (GCP-WD/NEDD1) and activating (CDK5RAP2) proteins. MZT1 can mediate the interaction by inducing a structural configuration change in the complex or linking the complex to the targeting/activating proteins. These interactions control MT nucleation in time and space throughout the cell cycle.

Conclusion

I. In cycling human cells MZT1 is required for γTuRC-dependent functions including centriole duplication.

II. MZT1 is a core subunit of the γTuRC that directly binds a hydrophobic motif present in the N-terminal extensions of GCP2, GCP3, GCP5 and GCP6.

III. The interaction with MZT1 is not required for the stability of the γTuRC, but it allows the recognition of fully assembled γTuRC.

IV. The association of MZT1 with the γTuRC primes the complex to be targeted by GCP-WD/NEDD1 to the centrosome, by enhancing interaction with GCP-WD/NEDD1.

V. The association of MZT1 with the γTuRC additionally primes the complex to be activated by CDK5RAP2, by enhancing interaction with the CM1 motif.

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