Dissecting γTuRC Recruitment and Microtubule Nucleation at MTOCs

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To all those who shined light along the way

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ABSTRACT

Microtubules form diverse arrays according to the cell type or the cell cycle stage to facilitate the context-specific functions of the cytoskeleton. One way by which cells achieve this is by restricting the formation of new microtubules (microtubule nucleation) to specific sites called microtubule organizing centers (MTOCs). In cycling cells, the bulk of microtubule nucleation is limited to the centrosome and/or the Golgi, despite the availability of the microtubule nucleator γ-tubulin ring complex (γTuRC) throughout the cytoplasm. Purified γTuRCs have been shown to have low nucleation potential *in vitro*. Taken together these observations suggest that additional factors are required to activate the nucleation of microtubules from γTuRCs specifically at MTOCs. Consistent with this are recent structural studies by our group and others that demonstrate the lack of symmetry of γTuRC to be a perfect template for nucleating microtubules. The pericentriolar material (PCM)-associated protein CDK5RAP2 is a potential candidate for enabling this activation through its evolutionarily conserved CM1 domain. Similarly, the microtubule polymerase XMAP215 has been implicated in facilitating nucleation from purified γTuRCs *in vitro*. The centrosomal Ninein-like protein (NINL) has been observed to recruit γTuRC to ectopic sites where the complex is able to nucleate microtubules.

In this thesis I analyze the factors that contribute to the recruitment of γTuRC to MTOCs and enabling microtubule nucleation at these sites. Firstly, I characterize NINL at the centrosome where I identify it as a subdistal appendage (SDA)-associated protein and analyze how its ability to induce microtubule nucleation at ectopic sites is linked to its ability to recruit the XMAP215 orthologue ch-TOG. Then I describe the contribution of ch-TOG in γTuRC recruitment and microtubule nucleation at the interphase centrosome and the Golgi. Lastly, I check how CM1 domain-mediated interactions play a role in centrosomal microtubule nucleation during interphase and mitosis. Overall, our results identify an important role of ch-TOG in γTuRC recruitment and microtubule nucleation at centrosomal and non-centrosomal MTOCs.

RESUM

Els microtúbuls formen matrius diverses segons el tipus cel·lular o l'etapa del cicle cel·lular per facilitar les funcions específiques del citoesquelet en cada context. Una manera en què les cèl·lules aconsegueixen això és restringint la formació de nous microtúbuls (nucleació de microtúbuls) a llocs específics anomenats centres organitzadors de microtúbuls (MTOC). A les cèl·lules ciclades, la major part de la nucleació de microtúbuls es limita al centrosoma i/o al Golgi, malgrat la disponibilitat del complex de l'anell de γ-tubulina nucleador de microtúbuls (γTuRC) a tot el citoplasma. S'ha demostrat que els γTuRC purificats tenen un potencial de nucleació baix in vitro. En conjunt, aquestes observacions suggereixen factors addicionals necessaris per activar la nucleació de microtúbuls a partir de γTuRCs específicament als MTOC. D'acord amb això, hi ha estudis estructurals recents del nostre grup i d'altres que demostren que la manca de simetria de γTuRC és una plantilla perfecta per nuclear microtúbuls. La proteïna CDK5RAP2 associada al material pericentriolar (PCM) és un candidat potencial per permetre aquesta activació mitjançant el seu domini CM1 conservat evolutivament. De la mateixa manera, la polimerasa de microtúbuls XMAP215 s'ha implicat recentment en la facilitació de la nucleació a partir de γTuRCs purificats in vitro. S'ha observat que la proteïna centrosomal de tipus nineïna (NINL) recluta γTuRC a llocs ectòpics on el complex és capaç de nuclear microtúbuls.

En aquesta tesi analitzo els factors que contribueixen al reclutament de γTuRC als MTOC i que permeten la nucleació de microtúbuls en aquests llocs. En primer lloc, caracteritzo NINL al centrosoma on l'identifico com una proteïna associada a l'apèndix subdistal (SDA) i analitzo com la seva capacitat d'induir la nucleació de microtúbuls en llocs ectòpics està relacionada amb la seva capacitat per reclutar ch-TOG, l'ortòleg de XMAP215. A continuació, descric la contribució de ch-TOG en el reclutament de γTuRC i la nucleació de microtúbuls al centrosoma en interfase i al Golgi. Finalment, comprovo com les interaccions mediades pel domini CM1 tenen un paper en la nucleació de microtúbuls centrosòmics durant la interfase i la mitosi. En general, els nostres resultats identifiquen ch-TOG com un actor central en el reclutament i l'activació de γTuRC als MTOC.

PREFACE

The work presented in this thesis aims to understand the key players in one of the fundamental processes in cells- the generation and organization of microtubule cytoskeleton. Using results from my thesis work, I have contributed to a research article and a mini-review that were published during the course of this thesis (also see Annex):

- Ali, A., Vineethakumari, C., Lacasa, C., & Lüders, J. (2023). Microtubule nucleation and γTuRC centrosome localization in interphase cells require ch-TOG. Nature Communications, 14(1), 289. https://doi.org/10.1038/s41467-023-35955-w

- Vineethakumari C and Lüders J (2022) Microtubule Anchoring: Attaching Dynamic Polymers to Cellular Structures. Front. Cell Dev. Biol. 10:867870. doi: 10.3389/fcell.2022.867870

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TABLE OF CONTENTS

INTRODUCTION

1

THE MICROTUBULE CYTOSKELETON:

Structure, Functions, Organization and Formation

1.1 Of cells and cytoskeleton

Ever since first observed by Robert Hooke more than three centuries ago, cells have remained as elements of fascination for anyone who tried to understand the mechanics of life and the living. Our understanding of the functioning of cells has evolved in response to the advancement of microscopic techniques that let us peer into depths of these tiny systems. Recent decades saw a sharp increase in our abilities to manipulate, observe, measure and analyze cellular activities in a multitude of ways helping us peel off the layers of mystery surrounding the question of how life functions. Yet, the complexity of the intricate cellular machineries that are shaped by millions of years of evolution continues to puzzle us.

The cytoskeleton is one among the many finest inventions of evolution, which did not simply provide a mechanical support to cells, but paved way for the structural and organizational complexity we now see in multicellular organisms. Cytoskeletal elements made it possible for cells to utilize and manipulate mechanical forces in a way that converted cells from simple diffusion systems to a higher order network with coordinated activities and communication.

Although not as profoundly complex as current eukaryotes, ancestral bacterial and archaeal populations maintained an elaborate cytoskeleton, which probably evolved in response to the need for mechanical forces required to physically divide a cell into two daughters. The earliest known element of cytoskeleton is the bacterial protein FtsZ. The rare presence of certain amino acids such as arginine or tryptophan in the FtsZ sequences among prokaryotes suggested a possibility that a functional FtsZ was evolved even before cells acquired a complete genetic code that include all the modern amino acids (Davis, 2002). The high degree of conservation of FtsZ between almost all known species of bacteria and archaea suggest how effective the use of this protein was since the early populations (Erickson, 2007). Interestingly, as eukaryotes evolved, emergence of an actin-based mechanism for cytokinesis rendered FtsZ free to undergo changes, resulting in one of the central elements of the eukaryotic cytoskeleton: tubulin.

The evolution from FtsZ to modern tubulin was quite drastic as only 10% of the sequence remain conserved. On the contrary, as microtubules, the polymerized version of tubulins, became ever more essential for basic cellular processes, tubulin ended up being one of the

most conserved proteins among eukaryotes with very little divergence (Doolittle, 1995; Keeling & Doolittle, 1996; Faguy & Doolittle, 1998; Doolittle & York, 2002; Erickson, 2007). Notably, the conserved sequences between FtsZ and eukaryotic tubulin include the amino acids associated with binding and hydrolysis of GTP, an essential energetic element for the proper functioning of microtubules (Erickson, 2007). Somewhat similar relationship exists between eukaryotic actin and its prokaryotic relative MreB, which assembles into actin-like filaments and is considered an evolutionary ancestor of actin. Actin and MreB show less than 15% sequence identity, among which the amino acids that carry out ATP binding activity are highly conserved (Van Den Ent et al., 2001; Doolittle & York, 2002). This is interesting considering how the earliest driving force of evolution of life was the use of purine nucleotides for the storage and conversion of energy needed for biological processes (*The vital question*, Nick Lane).

It is curious to note that based on the activity of actin-like proteins found in bacteria, earlier relatives of actin might have performed the function of segregating DNA molecules between daughter cells (Becker et al., 2006) and tubulin ancestor FtsZ was used for cytokinesis, whereas modern eukaryotes completely switch these roles by using tubulin-based microtubules for chromosome segregation and actin-based system for cytokinesis.

This thesis is an attempt to understand the complex organizational system of the microtubule cytoskeleton seen in modern eukaryotes. Specifically, it addresses what components and activities are involved in generating new microtubules in human cells. In the following chapters I will discuss in detail the structural and functional features of microtubules and how cells regulate their formation and organization.

1.2 Microtubules

Microtubules are elements of the cytoskeleton, and as the word suggest, they act as the skeletal system within the cells by forming an elaborate network spanning the entire cellular interior, helping maintain the cell shape and arrange the intracellular organelles in place. They enable and regulate intracellular transport by paving a track for motor proteins that carry cargo molecules or vesicles. At the same time, they are flexible enough to allow motility and cell shape changes or adopt diverse architectural arrangements necessary for a specific cell type or cell cycle stage, such as the formation of a bipolar spindle during cell division.

One central feature of microtubules is their dynamicity. Microtubules are long tubular structures formed by the polymerization of tubulin protein units. They have the intrinsic property to frequently switch between phases of growth and shrinkage and this is additionally regulated by a multitude of cellular cues including, but not limited to, the availability of tubulin subunits and the activity of microtubule-stabilizing or -destabilizing factors This intrinsic feature of microtubules, known as dynamic instability, is essential for much of their functions by ensuring flexibility to adapt to the needs of the cell.

Microtubules are composed of two types of tubulin subunits called α- and β-tubulin. They exist as obligate heterodimers in cells and their longitudinal interactions generate a polarized protofilament chain where β-tubulin of one heterodimer interacts longitudinally with the α-tubulin of the subsequent heterodimer (**Figure I1.1a**). Interactions between α-tubulins or β-tubulins usually occur laterally, between neighboring tubulins in adjacent protofilaments, and contribute to the formation of a cylindrical microtubule (**Figure I1.1b**). One inherent feature resulting from such an arrangement of two tubulin subtypes is the intrinsic polarity of microtubules where α-tubulins are exposed at one end and the other is marked by exposed β-tubulins, defining the so-called minus- and plus-end, respectively. Such a polarity is recognized by microtubule associated proteins (MAPs) and motor complexes allowing directed motion along the microtubule or binding to a specific end (Nogales et al., 1999; Kollman et al., 2011).

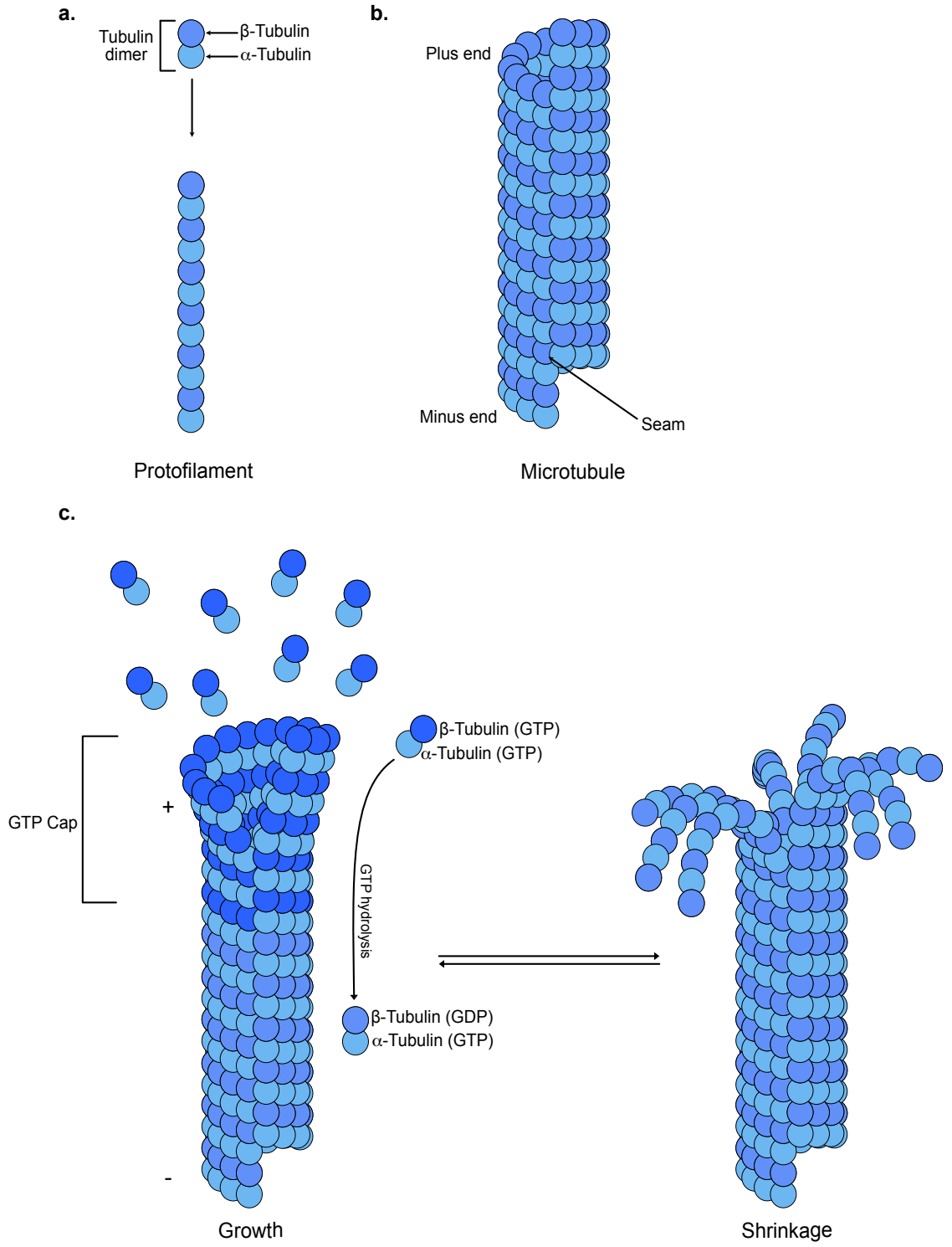


Figure I1.1: Microtubule structure and dynamics
a. Longitudinal interactions between tubulin dimers forms a protofilament.
b. Lateral association of protofilaments generate a cylindrical microtubule with intrinsic polarity.
c. Microtubules frequently switch between stages of growth and shrinkage known as dynamic instability.

The dynamicity of growing microtubule ends is driven by the binding and hydrolysis of GTP molecules to the tubulin dimers. Although both α- and β-tubulins bind GTP, only the GTP molecule bound by β-tubulin undergoes hydrolysis to become GDP. However, GTP hydrolysis occurs only after incorporation of tubulin dimers into the microtubule lattice. In their GTP-bound state, which promotes a straight conformation, heterodimers get added to microtubule plus-ends. After incorporation, the GTP bound by β-tubulin in the trailing microtubule shaft eventually gets hydrolyzed, upon which the protofilaments tend to prefer a curved architecture. Curved protofilaments are unstable and can 'peel off', resulting in microtubule depolymerization. In growing microtubules, this is prevented by the presence of a "GTP cap", layers of GTP-bound tubulin dimers at the growing tips. Loss of this cap by GTP hydrolysis, which occurs stochastically, will result in depolymerization or catastrophe (**Figure I1.1c**). One factor that determines the switch between these states is the availability of GTP-bound tubulin dimers to be continuously added so the ends remain GTP capped (Mitchison & Kirschner, 1984a; Drechsel et al., 1992; Desai & Mitchison, 1997; Alushin et al., 2014).

Microtubules formed from purified tubulin *in vitro* have been observed to contain varying numbers of protofilaments ranging from 11 to 16, but in cells, a strict number of 13 protofilaments is maintained (Tilney et al., 1973; Evans et al., 1985; Chrétien & Wade, 1991; Nogales et al., 1999; Kollman et al., 2011). Interestingly, only microtubules with 13 protofilaments show an unskewed lattice with straight protofilaments, while other lattice architectures reveal twisted protofilament arrangement (Sui & Downing, 2010). It is possible that in cells, avoiding such twisted protofilament in the lattice is essential for the processive movement of motor proteins, resulting in the exclusive use of 13 protofilament microtubules. The lateral contacts between the neighboring protofilaments are mediated through interactions between the same type of tubulin subunits, with one exception. The helical pitch of the microtubule lattice leads to a vertical offset of 3 monomers between the first and last of the 13 tubulins that constitute one turn of the microtubule wall (called the 3-start helix structure). This results in a seam, at which the lateral interactions occur between α- and a β-tubulin subunits (**Figure I1.1b,c**) (McEwen & Edelstein, 1980; Mandelkow et al., 1986).

In addition to utilizing the intrinsic dynamicity and polarity of microtubules, cells go one step further to make microtubules more versatile by manipulating the tubulin subunits themselves. The use of differentially expressed isoforms of both α- and β-tubulins, combined with a plethora of post-translational modifications create a 'tubulin code' that allows the modulation of microtubule properties to a much finer degree (Gadadhar et al., 2017; Janke & Magiera, 2020). The tubulin genes are highly conserved across species yet there exist multiple genes for both α- and β-tubulin subunits, allowing variations to evolve (Ludueña, 2013; Ludueña & Banerjee, 2008).

Humans have nine different genes each for α- and β-tubulins that produce isotypes with subtle variations, mostly at the C-terminus. The C-terminal ends of tubulins incorporated in the microtubule wall extend outwards as flexible tails, facilitating interactions with MAPs and presenting sites for post translational modifications. Thus, variations in this region between isotypes can control differential binding of MAPs. Moreover, the high degree of sequence conservation in the core structure allows different tubulin subtypes to intermix and create a mosaic microtubule (Lewis et al., 1987). It is not clear whether such a mix of tubulin isotypes in a microtubule influences the mechanical properties and assembly dynamics, however, preferences over certain types of tubulin isotypes in specific scenarios have been observed. Ciliary axonemes and neuronal microtubules are some examples that selectively utilize specific β-tubulin isotypes (Joshi & Cleveland, 1989; Raff et al., 2008).

Tubulin molecules are modified post-translationally in many different ways. Some well-studied modifications include acetylation, which occurs in the lumen-facing side of α-tubulin (L'Hernault & Rosenbaum, 1985), tyrosination, which involves a tRNA-independent addition of a tyrosine amino acid to the C-terminal tail of tubulins (Arce et al., 1975) and detyrosination, the removal of the terminal tyrosine residue from tubulin that is either present in the expressed sequence or added by a tyrosination process (Hallak et al., 1977). Upon detyrosination, tubulins are sometimes subjected to further removal of 2 or 3 terminal residues resulting in Δ2 or Δ3 tubulin, respectively, which prevents any subsequent tyrosination (Aillaud et al., 2016). Another form of modification is the addition of multiple glutamate or glycine residues to the C-terminal ends, known as polyglutamylation or polyglycylation (Eddé et al., 1990; Redeker et al., 1994). A variety of further modifications

including methylation, sumoylation and several others play key roles in modulating tubulin properties thereby controlling microtubule functions (Janke & Chloë Bulinski, 2011; Gadadhar et al., 2017; Janke & Magiera, 2020).

1.3 Microtubule Organizing centers

Cells depend on specific spatial arrangements of microtubules to ensure proper organization of the cellular interior. In addition, to efficiently take advantage of the inherent polarity and dynamicity of microtubules for particular functional roles, it is important to ensure a specific shape of the microtubule network with appropriate orientations of individual microtubules. Such microtubule arrays vary according to the cell cycle stage or the cell type, and their formation, maintenance and reorganization is precisely controlled. One way by which cells gain control over the microtubule arrangements is by limiting their formation and positioning spatially and temporally. Formation of new microtubules is known as 'microtubule nucleation'. Microtubule organizing centers (MTOCs) are the designated cellular sites where new microtubules are nucleated and anchored. Microtubules are typically arranged with their minus-ends stabilized and anchored at the MTOCs, while the dynamic plus-ends grow out and explore the cytoplasm.

Microtubules that grow out from a central MTOC result in the formation of a radial array, typically seen in proliferating cells during interphase with the aptly named centrosome acting as the MTOC. Dividing cells organize a bipolar microtubule array, the mitotic spindle, with minus-ends clustered at the two spindle poles and the dynamic plus-ends at the center interacting with kinetochores to align the chromosomes on the metaphase plate. Differentiated cells display a wide range of microtubule arrays emanating mostly from distinct non-centrosomal MTOCs (**Figure I1.2**).

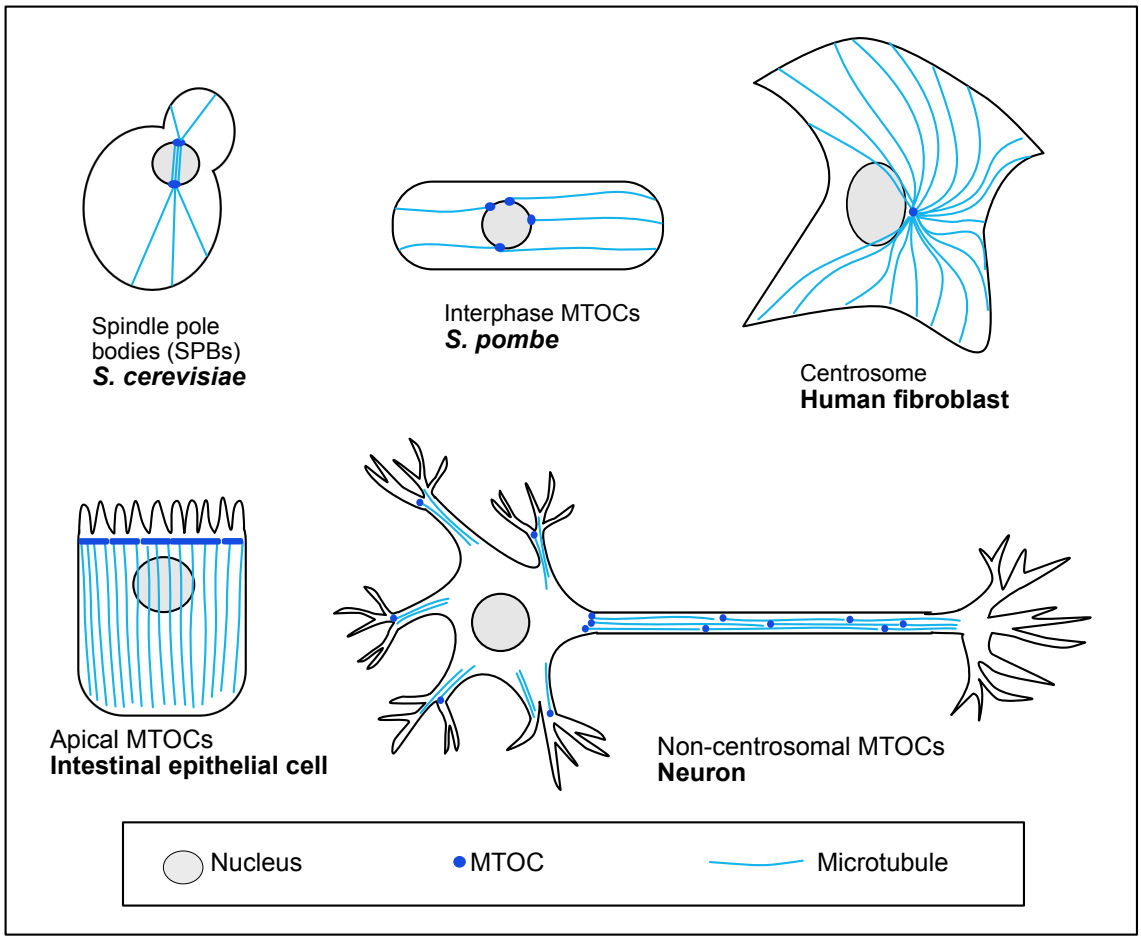


Figure I1.1: Microtubule organization by MTOCs in different cell types

Examples of different microtubule organizations found in various cell systems. Type of MTOCs in each system specified below (Adapted from Sanchez and Feldman, 2017.)

Spindle pole bodies (SPBs)

The spindle pole bodies (SPBs) found in budding and fission yeasts, which are analogous to the centrosome in animal cells are used to organize the mitotic spindle in these organisms. The closed mitosis in budding yeast is mediated by a nuclear envelope bound SPB that organize separate populations of nuclear and cytoplasmic microtubules. The former makes up the spindle while the latter interacts with the cell cortex for positioning the nucleus (Knop et al., 1999). Unlike budding yeast, fission yeast harbors interphase MTOCs at the nuclear envelope in addition to the SPB that nucleate and organize microtubules along the cell axis (Hagan, 1998; Drummond & Cross, 2000; Sawin et al., 2004; Sawin & Tran, 2006; W. Liu et al., 2019). During mitosis, the duplicated SPBs move towards opposite poles and organize

the spindle (Hagan, 1998; Janson et al., 2005; Sawin & Tran, 2006). Higher eukaryotes utilize a variety of MTOCs, the most prominent of which is the centrosome.

Centrosome

Centrosomes are easily recognizable in cycling cells as the center of a radial microtubule array in interphase or at the spindle poles of dividing cells. It is the major and best studied MTOC in animal cells. Despite not being membrane-bound like other organelles, centrosomes maintain their distinct composition, which includes a pair of microtubule-based cylinders called centrioles surrounded by a matrix of proteins that constitute the pericentriolar material (PCM). Centrosomes are complex organelles that carry out diverse functions including microtubule formation, anchoring, cilia assembly and at times they also act as a hub for signal transduction within cells (Arquint et al., 2014; Conduit et al., 2015). The unique structural features and protein composition of centrosomes are key to their function.

Notably, centrosomes undergo cyclical changes linked to cell cycle progression that include essential processes such as centrosome duplication and maturation. A typical G1 centrosome consist of 2 centrioles connected by a flexible linker (**Figure I1.3**) (Agircan et al., 2014; Conduit et al., 2015).

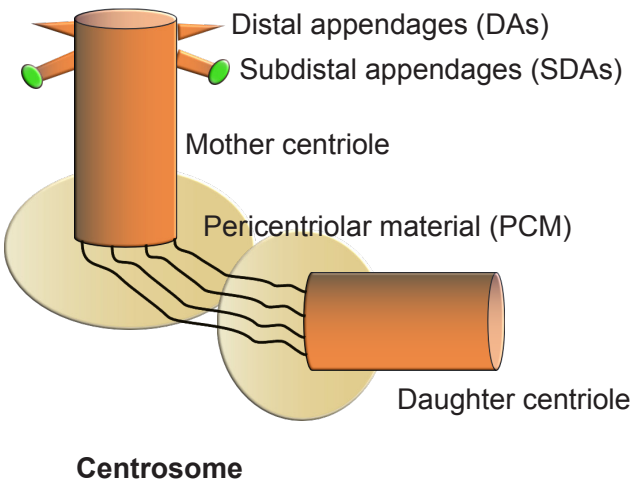


Figure I1.2: Centrosome structure

Cartoon of a centrosome in G1 phase, depicting sub-centrosomal compartments

The cylindrical centriole structure is formed by an arrangement of nine sets of triplet microtubules and has a length of about 0.5 μm and a width of about 0.2 μm (Azimzadeh & Bornens, 2007; Agircan et al., 2014). One of the two centrioles, the mother centriole, is older and can be distinguished by the presence of distal and subdistal appendages. Distal appendages consistently follow the nine fold symmetry of centrioles, whereas the subdistal appendages (SDAs) vary in number between cell types (Hall & Hehnly, 2021; Tischer et al., 2021). Distal appendages contribute to cilia assembly by docking the mother centriole at the plasma membrane (Tanos et al., 2013). On the other hand, SDAs are the designated sites that anchor centrosomal microtubules (Bornens, 2002; Tischer et al., 2021). Notably, some SDA associated proteins such as Ninein and CEP170 additionally also localize to the proximal ends of both centrioles. Although the functional aspects of SDA components at proximal centriole ends are not well studied, they have been implicated in maintaining centriole cohesion along with their recruitment factor C-Nap1 (Mazo et al., 2016; Chong et al., 2020). Interestingly, in addition to its MTOC activity, centrosome has been proposed to act as a hub that coordinate signaling activities within the cell (Arquint et al., 2014) and recent observations indicate a role for the SDA component CEP170 in signaling associated with DNA double strand brake repair (Rodríguez-Real et al., 2023).

The older centriole is called mother centriole because it templated the assembly of the younger daughter centriole in the previous cell cycle. The two centrioles are connected through a proteinaceous linker at their proximal ends and this is also the region where the PCM proteins are associated. A common feature of most PCM proteins is their extensive coiled-coil domains that promote higher order protein-protein interactions as seen in core PCM components such as CEP63, CEP152, Pericentrin (PCNT) and CDK5RAP2. While it was presumed that PCM is an amorphous mixture of such scaffold proteins (Robbins et al., 1968), later super resolution analyses of interphase PCM revealed a highly organized and layered distribution of proteins (Fu & Glover, 2012; Lawo et al., 2012; Mennella et al., 2012; Sonnen et al., 2012; Woodruff et al., 2014; K. S. Lee et al., 2021). In interphase, the bulk of the microtubules are formed within the PCM surrounding the mother centriole (Gould & Borisy, 1977).

At the onset of S phase, both centrioles start to accumulate factors needed for the biogenesis of new centrioles. Centriole duplication is initiated by the formation of procentrioles on the

surface of both the existing centrioles, in a perpendicular orientation, converting the younger centriole into a new mother. The kinase Plk4 is the major player in the regulation of centriole duplication process (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Azimzadeh & Bornens, 2007). A nine-fold cart wheel structure made by SAS6 provides the template for the symmetric synthesis of new centrioles (Nakazawa et al., 2007; Kitagawa et al., 2011). Once formed, these new daughter centrioles remain attached (engaged) to their respective mothers until the end of the following mitosis. During the G2 phase the new mother centriole (the younger centriole from the G1 phase) starts to accumulate components like OFD1 and ODF2 that will lay the foundation for the assembly of appendages, although this assembly is not completed until entry to the next G1 (Huang et al., 2017; Sullenberger et al., 2020; Tischer et al., 2021). At the same time, some of the existing appendage structures from the older mother are lost as the cell prepares for mitosis (Sullenberger et al., 2020; Tischer et al., 2021; Hall & Hehnly, 2021). In late G2 before mitotic entry, the flexible linker between the two mothers is disassembled, allowing the formation of two independent centrosomes, that move away from each other to form the two spindle poles.

Before entry to mitosis, the PCM undergoes a dramatic expansion in size in a process termed centrosome "maturation", which ensures robust spindle assembly. The phosphorylation of PCM scaffold proteins like PCNT and CDK5RAP2 by mitotic kinases such as Plk1 increases their interactions and association with the centrosome, promoting their accumulation around the centrioles (Haren et al., 2009; Kim & Rhee, 2014; Joukov et al., 2014; K. S. Lee et al., 2021). Such an expanded PCM allows the assembly of microtubules at a much higher rate than seen in interphase, ensuring the supply of dense bundles of microtubules that make up the spindle fibers. Curiously, this accumulation of PCM proteins during centrosome maturation results in a less organized matrix in the outer PCM region compared to the layered structure of interphase PCM, which remains present throughout mitosis in the region closest to the centriole wall. The expanded mitotic PCM does not only generate a large number of microtubules, but also clusters and anchors their minus-ends, resisting the mechanical forces exerted by the spindle (K. S. Lee et al., 2021). Towards late mitosis, the mother centrioles at each spindle pole continue to incorporate components of the appendages and, upon exit from mitosis and cell division, each daughter cell receives a pair of centrioles. This involves the disengagement of newly formed daughters from the surface of their respective mothers and establishment of the flexible fibrous protein linker between

mother and daughter. The new daughters recruit their own PCM (centriole-to-centrosome conversion) and are ready to generate procentrioles in the next S phase (Piel et al., 2000; W.-J. Wang et al., 2011; Agircan et al., 2014).

In cells that do not proceed to the next cycle, such as many differentiated cells or quiescent cells (also called the G0 phase), the centrioles get converted into basal bodies that assemble cilia. Cilia are specialized membrane protrusions that perform either sensory or motility functions depending on the type of cilia, called primary or motile cilia. At the core of the cilia is a microtubule-based axoneme, which is an extension of the mother centriole that gets anchored to the plasma membrane using distal appendages. Primary cilia are crucial signaling organelles during vertebrate development. Mutations that affect primary cilia formation or function lead to a range of developmental defects (Goetz & Anderson, 2010; Lovera & Lüders, 2021).

Non-centrosomal MTOCs

In some cell types, even when the centrosome remains the dominant MTOC, the Golgi apparatus contributes significantly to organizing the microtubule network (Paz & Lüders, 2018; Wu et al., 2016). The presence of such a non-centrosomal MTOC may be beneficial under conditions that compromise centrosomal activity. Indeed, the Golgi has been shown to compensate for centrosome loss by enhanced MTOC activity (Wu et al., 2016; Gavilan et al., 2018). Similar to centrosomes, the Golgi has the ability to both nucleate and anchor microtubules (Efimov et al., 2007; Rivero et al., 2009; Wu et al., 2016; Gavilan et al., 2018). Given that a major role of the Golgi is vesicle sorting and secretion, such an intimate link with microtubule organization may facilitate microtubule-based transport of vesicles in specific directions. In cycling cells, the MTOC activity of the Golgi is restricted to interphase. Golgi undergoes fragmentation during mitosis which facilitates the segregation of Golgi compartments between daughter cells and this coincides with a loss of MTOC activity at the Golgi (Shorter & Warren, 2002; Wei & Seemann, 2009; X. Zhu & Kaverina, 2013). Regaining of the MTOC activity after mitosis contributes to Golgi reassembly as microtubule-based transport of dispersed Golgi stacks allows for their clustering and fusion into a larger Golgi ribbon (P. M. Miller et al., 2009; Vinogradova et al., 2012). Additionally, interaction with the centrosomal microtubules allows positioning of this reassembled Golgi in proximity of the

centrosome (P. M. Miller et al., 2009; N. Nakamura et al., 2012; X. Zhu & Kaverina, 2013; Rios, 2014).

The Golgi-associated microtubules can be either assembled there or be of centrosomal origin and transported to the Golgi with the help of minus-end stabilizing proteins such as CAMPSAP2 (Keating et al., 1997; Jiang et al., 2014; Wu et al., 2016). In cycling cells, the Golgi is usually secondary to the centrosome as an MTOC, whereas most differentiated cells downregulate the MTOC activity at the centrosome and depend more strongly on the Golgi MTOC or other non-centrosomal MTOCs (Paz & Lüders, 2018; Sallee & Feldman, 2021). An interesting aspect of Golgi-associated microtubules is their ability to dictate cell polarity.

Contrary to the radial array of microtubules organized by the centrosome, the ribbon shape of the Golgi helps to form a polarized array of microtubules on one side of the cell, where the Golgi is located. In addition, Golgi stacks themselves are polar in nature, with the cis-Golgi compartment facing the centrosome and nucleus and the trans-Golgi membranes facing the cell periphery. Based on current understandings, microtubules are nucleated at the cis side, but require stabilization from trans-Golgi associated factors for successful growth (Wu et al., 2016). Therefore, a cis to trans polarity of Golgi derived microtubules is established where plus-ends face only one side of the cell thereby polarizing the cell (**Figure I1.4**). In migratory cells, the side faced by the Golgi ends up being the leading edge with actively growing microtubule ends and membrane protrusions while the other side follows as trailing edge (Vinogradova et al., 2009; Ravichandran et al., 2020; Sallee & Feldman, 2021).

Apart from migration, in many differentiated cell types polarized microtubule networks are utilized for morphogenesis into diverse cellular forms as seen for example in muscle cells or particularly in neurons, where it is essential for neurite outgrowth and branching. In addition, polarized microtubule arrays mediate the transport of secretory vesicles in many cell types (Ori-McKenney et al., 2012; Oddoux et al., 2013; X. Zhu et al., 2015; Sallee & Feldman, 2021).

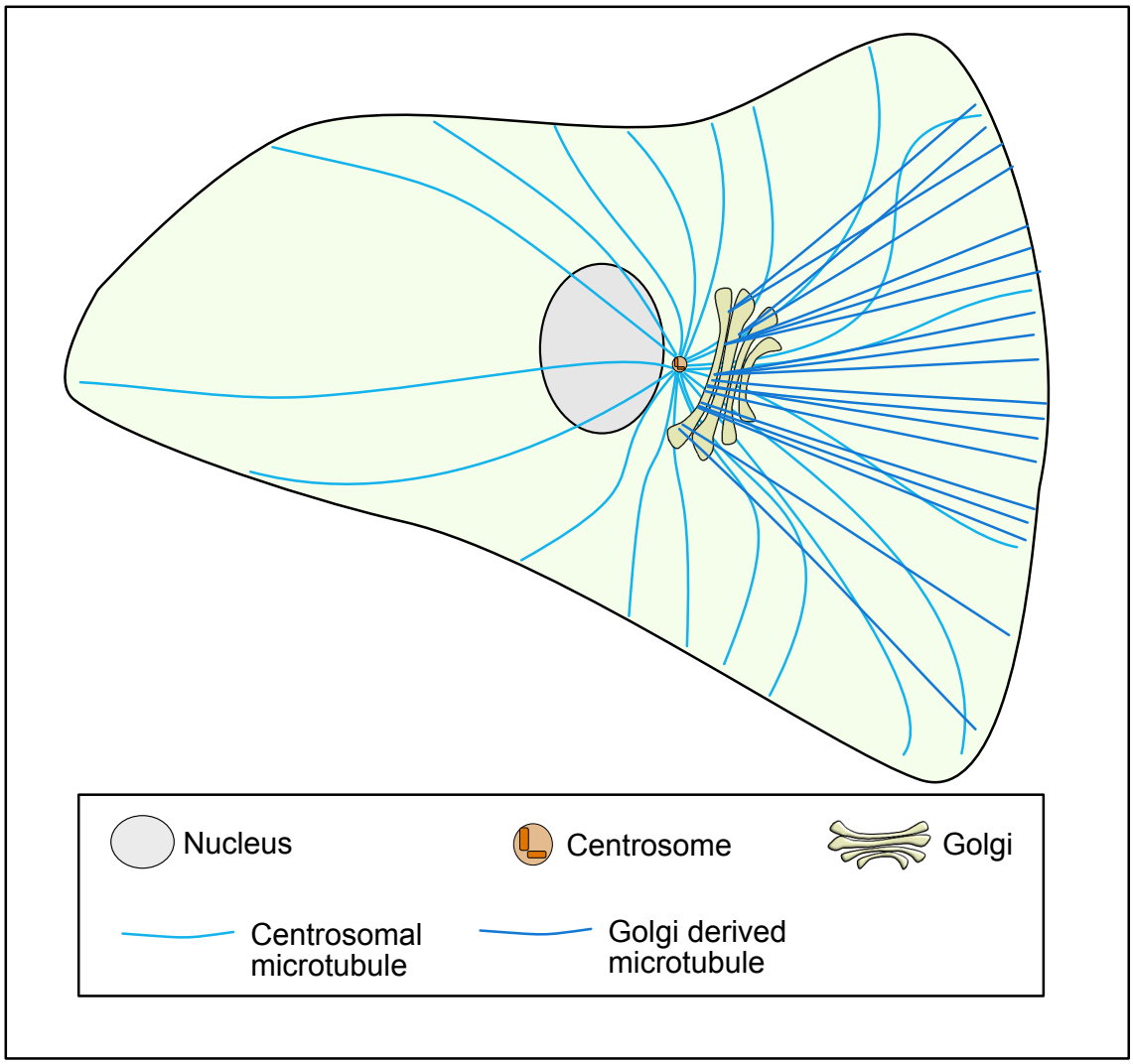


Figure I1.3: Golgi promotes polarized microtubule networks

Cartoon representation of a polarized microtubule network formed by Golgi in migratory cells

Aside from the Golgi, a wide variety of MTOCs exist in cells, most of which become relevant as cells undergo differentiation and gradually downregulate centrosomal MTOC activity. Such a transition from centrosomal to non-centrosomal MTOCs helps cells to make use of polarized microtubules networks of diverse organizational shapes. The nuclear envelope-associated MTOC seen in muscle cells, the apical and junction-associated MTOCs that generate apico-basal microtubule arrays in epithelial cells, and the endosome-associated MTOCs at the dendritic ends that help generate minus-end-out microtubules in *Drosophila* and *C. elegans* neurons are some examples among many others (Bellett et al., 2009; Feldman

& Priess, 2012; Muroyama et al., 2016; Paz & Lüders, 2018; Sallee & Feldman, 2021; A. M. Tassin et al., 1985).

Interesting to note here is that microtubules themselves can act as MTOCs whereby new microtubules are nucleated from the lattice of an existing microtubule creating microtubule branches (Petry et al., 2013). Such a mechanism helps to increase the density of microtubules in the mitotic spindle to allow robust metaphase spindle assembly and chromosome segregation (Goshima et al., 2008; Prosser & Pelletier, 2017). Similar branching microtubules are also seen in the interphase cortical microtubule array of plant cells (Chan et al., 2003, 2009; Murata et al., 2005) and fission yeast interphase microtubule bundles (Janson et al., 2005). In addition to nucleation, microtubules display some level of self-organization with the help of microtubule associated proteins (MAPs), which help to stabilize microtubules and regulate growth rates. More importantly, the coordinated actions of various motor proteins that can slide microtubule against each other allow for the arrangement of microtubules with specific polarity and for the clustering and focusing of their minus-ends even in the absence of a centrosomal MTOC as seen for example, in mammalian oocytes (Dammermann et al., 2003; M. Martin & Akhmanova, 2018; Sallee & Feldman, 2021; So et al., 2022; Henkin et al., 2023). Similarly, microtubule dynamic instability combined with treadmilling induced by a balanced addition and removal of subunits at microtubule ends promote microtubule organization in plant cells (Shaw et al., 2003; Elliott & Shaw, 2018).

Anchoring microtubules at MTOCs

As mentioned earlier, MTOCs are generally attributed to two main functions. One is the formation of new microtubules, which will be discussed in detail in the following sections. The second is to anchor the microtubules stably to generate spatial patterning of the microtubule network (Vineethakumari & Lüders, 2022). By contrast to *in vitro* assembled microtubules that exhibit dynamicity at both ends, in cells, microtubule minus-ends are usually bound and anchored allowing the plus ends to grow and explore the cellular interior. Thus, anchoring needs to involve stabilization of the minus ends for example by capping. In addition, microtubules experience and produce substantial amounts of pushing and pulling forces owing to their dynamicity, association with motor proteins, etc. Hence robust

anchoring of microtubules involves resisting such mechanical forces, but also being flexible for any reorganizations when necessary.

At the centrosome, the SDAs are the designated sites for anchoring microtubules. As most of the microtubules are nucleated at the PCM, it is not clear if and how they are transferred to the SDAs for anchoring. At the SDAs, Ninein (NIN) is the major component linked with anchoring (Bouckson-Castaing et al., 1996; Mogensen et al., 2000; Dammermann & Merdes, 2002; Ou et al., 2002; Delgehyr et al., 2005; C.-C. Lin et al., 2006). The consistent presence of PCM-associated microtubules would indicate many microtubules are anchored at the PCM as well, possibly within the layers of PCM proteins, but as NIN is also localized at the proximal ends of centrioles, it could be contributing to anchoring microtubules at this site as well. The presence of NIN also at non-centrosomal MTOCs in differentiated cells such as the apical MTOCs in epithelial cells or the nuclear envelope-based MTOC in muscle cells is consistent with its role as an anchoring factor (Moss et al., 2007; S. Wang et al., 2015; Goldspink et al., 2017). In many such non-centrosomal MTOCs CAMSAPs play an integral role in stabilizing the minus ends facilitating anchoring (N. Tanaka et al., 2012; Jiang et al., 2014; Nashchekin et al., 2016; Noordstra et al., 2016; Toya et al., 2016; Atherton et al., 2017). While the centrosomal microtubules seem to be mainly associated with the PCM and the SDAs, another, less studied site of anchoring microtubules is the central region of the centriolar cylinder, where anchoring is mediated by FSD1, which forms a ring around the centriole and directly interacts with microtubules (Tu et al., 2018). However, it is possible that this is not a permanent anchoring site but rather a transitory site when transferring PCM-nucleated microtubules to the SDAs for stable anchoring.

1.4 Microtubule nucleation

In cells, microtubule formation and organization are highly regulated by MTOC-associated factors, whereas purified α-β-tubulin dimers under the right temperature and buffer conditions, can spontaneously assemble microtubules *in vitro*, with GTP and Mg^{2+} being major requirements (J. C. Lee & Timasheff, 1977; Roostalu & Surrey, 2017).

Given the polymer nature of microtubules, it is straightforward to assume how the availability of constituent subunits directly contributes to the growth rate. Understandably, the concentrations of α-β-tubulin dimers in the solution can determine whether the microtubules favor growth or shrinkage. *In vitro* experiments demonstrate a linear relationship between the microtubule growth rate and tubulin concentrations of a dynamic microtubule (Mitchison & Kirschner, 1984a; Drechsel et al., 1992; Wieczorek et al., 2015). Such a linear relationship predicts a minimal concentration to allow polymerization, below which, the polymer will undergo shrinkage, known as the 'critical concentration'. While this is true for a microtubule already in growth, the initial formation of the first detectable microtubule structure requires a much higher concentration of α-β-tubulin dimers. (Mitchison & Kirschner, 1984b; Walker et al., 1988; Wieczorek et al., 2015).

Such a need for higher tubulin concentration is mostly attributed to the complex 3-dimensional organization of microtubules that requires the lateral association of multiple protofilaments. This implies that rather than the simple linear addition of subunits, several longitudinal and lateral interactions between tubulins need to occur simultaneously to form a stable minimal structure that would then allow elongation. Thus, any assembly process would go through multiple, relatively unstable oligomeric intermediates before reaching a critical nucleus that can support growth, creating a kinetic barrier for this process (**Figure I1.5a**). This kinetic barrier can be observed as a lag phase in the sigmoidal curve of microtubule nucleation assays with respect to time (**Figure I1.5b**).

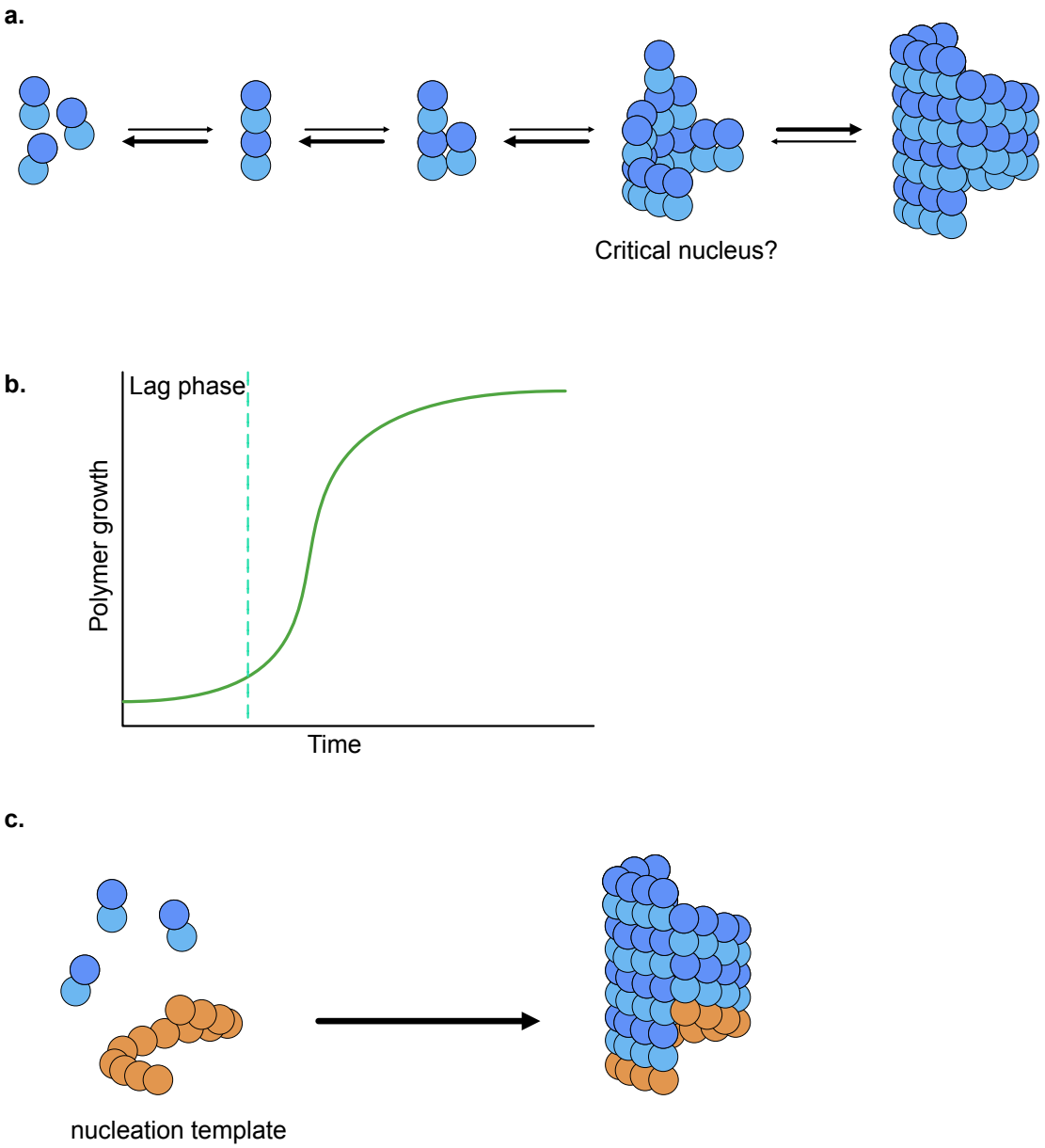


Figure I1.4: Kinetics of microtubule nucleation

a. A hypothetical scheme for the spontaneous microtubule assembly through the association of tubulin dimers. Thickness of the arrow marks indicates the direction that is kinetically favored. Initial tubulin olgomers are less stable making them dissociate easily, but once a critical nucleus is formed, further growth intro long microtubules is favored.
b. A typical sygmoid curve observed in nucleation assays from tubulin dimers. The lag phase represents the slow kinetics until the critical nucleus is formed.
c. Intermediate tubulin oligomers can be skipped by having a preformed template
(Adapted from Roostalu and Surrey, 2017)

Although this kinetic barrier cannot be completely eliminated, it can be reduced by increasing the tubulin concentrations, which promote the probability of interactions, or by stabilization of the intermediate oligomers (Hyman et al., 1992; Wieczorek et al., 2015; Roostalu & Surrey, 2017). As described in the previous section, hydrolysis of tubulin-associated GTP to GDP makes the oligomers less adapted for the straight microtubule lattice. Therefore, reducing the rate of hydrolysis would allow the formation of more stable intermediates as seen by the use of the non-hydrolysable GTP analogue GMPCPP (J. C. Lee & Timasheff, 1977; Wieczorek et al., 2015). Use of glycerol in microtubule preparations *in vitro* has also shown to promote the nucleation process (J. C. Lee & Timasheff, 1977).

Similarly, several microtubule-associated proteins are able to stabilize the intermediates and promote the cooperative interactions between tubulin dimers thereby enhancing nucleation. Given that the cellular concentrations of tubulins are not sufficient to reduce the kinetic barrier and allow spontaneous nucleation, such MAPs might contribute to nucleation in cells. Alternately, the kinetic barrier could be overcome by the presence of a preassembled stable template to act as a nucleus that allows further growth (**Figure I1.5c**) (Roostalu & Surrey, 2017). The use of such a preformed template could have an added advantage of dictating the microtubule geometry by restricting the number of protofilaments incorporated into the lattice, explaining the observed 13-protofilament structure of cellular microtubules. Combining this templated mechanism of nucleation with additional stabilization from MAPs could be an efficient way to overcome the kinetic barrier of microtubule nucleation in cells. Indeed, such a nucleator complex that forms a template for microtubule nucleation has been identified and localized to centrosomes and to all non-centrosomal MTOCs analyzed so far.

1.5 The nucleator complex

γ-tubulin: the essential component

In addition to the constituent α- and β-tubulin subunits, microtubule nucleation in cells requires a third type of tubulin, γ-tubulin, which associates with the gamma complex proteins (GCPs) to form a multi subunit γ-tubulin complex (Farache et al., 2018; Tovey & Conduit, 2018).

γ-Tubulin was first discovered in *Aspergillus nidulans* and identified as an essential component for microtubule function at the spindle pole body (Weil et al., 1986; C. E. Oakley & Oakley, 1989; B. R. Oakley et al., 1990). The sequence similarity in the N-terminal region of γ-tubulin with that of α- and β-tubulins suggested that microtubule nucleation might involve the interaction of γ-tubulin with α-β-tubulin dimers similar to the contact between the dimers in a microtubule lattice (B. R. Oakley et al., 1990).

Identification of γ-tubulin in other organisms from budding yeast and plants to human cells led to establishing it as an essential and universal component of MTOCs and a necessary factor in microtubule assembly (Stearns et al., 1991; Zheng et al., 1991; Joshi et al., 1992; Horio & Oakley, 1994; B. Liu et al., 1994; Sunkel et al., 1995). An interesting breakthrough was the discovery made in *Xenopus* egg extracts and *Drosophila* cells that γ-tubulin is present in the PCM as part of a multi-subunit complex that has a ring like shape (Joshi et al., 1992; Stearns & Kirschner, 1994). In purified centrosomes, these rings remained associated with the PCM in the absence of microtubules. Upon regrowth, γ-tubulin was found to be associated with the minus ends of newly formed microtubules (Moritz et al., 1995), suggesting its role in forming a nucleator complex for centrosomal microtubule nucleation. Earlier models suggested γ-tubulin molecules might interact longitudinally to form a protofilament like structure, which would then laterally interact with α-β- tubulin dimers to help form the protofilaments of microtubules (Erickson, 2000). Such a model was later discarded as observation of γ-tubulin-containing rings supported the idea that γ-tubulin is part of a complex that recapitulates the circular symmetry of a microtubule cross-section,

thereby providing a template for nucleation (Moritz et al., 1995; Zheng et al., 1995; Moritz et al., 2000).

The γ-tubulin complex purified from *Xenopus* egg extract, named as γ-tubulin ring complex (γTuRC) for its shape, demonstrated the ability to nucleate microtubules at a rate higher than the spontaneous nucleation, as well as to bind to and stabilize the minus ends of preformed microtubules (Zheng et al., 1995). The essential role of γ-tubulin complexes in microtubule nucleation at the centrosome was established through complementation studies of salt-stripped *Drosophila* centrosomes, which also identified the presence of two different complexes that contain γ-tubulin, one of 240KDa in size and one of roughly 3 MDa (Moritz et al., 1998). The larger complex displayed the characteristic ring structure of the γTuRC as observed in *Xenopus* egg extracts. The smaller γ-tubulin complex (γTuSC) was identified as a sub-complex of the γTuRC, and was shown to have much weaker nucleation potential compared to the larger complex (Moritz et al., 1998; Oegema et al., 1999).

In addition to γ-tubulin, additional components that are unrelated to tubulins make up the γ-tubulin complex, the most prominent of which are members of the GCP family. Multiple GCP family proteins are involved in the formation of γ-tubulin complexes. These proteins are mainly characterized by the presence of an amino terminal grip1 motif and the carboxy terminal grip2 motif (Gunawardane et al., 2000), which are highly conserved between the members of the family. The N-terminal grip1 motif facilitates interaction between the GCPs (Farache et al., 2016) while the grip2 region is required for binding to γ-tubulin molecules (Knop, 1997b; O. C. Martin et al., 1998; Kollman et al., 2008).

Budding yeast has only two GCP family proteins, Spc97 and Spc98, which were first identified to be forming a complex with γ-tubulin and facilitate microtubule organization at the spindle pole body (SPB) (Geissler et al., 1996; Knop, 1997a, 1997b). Detailed analysis of the proteins from purified human γ-tubulin complex identified the human GCP2 and GCP3 as orthologues of Spc97 and Spc98 based on sequence similarity (Murphy et al., 1998). Identification of orthologues from other organisms revealed these proteins to be highly conserved and indispensable for cell viability (Knop, 1997b; O. C. Martin et al., 1998; Gunawardane et al., 2000; Vardy, 2000; Colombié et al., 2006; J. Liu & Lessman, 2007; Seltzer

et al., 2007; Xiong & Oakley, 2009; Guillet et al., 2011; Oegema et al., 1999). Experiments in fission yeast demonstrated that GCP2 and GCP3 show high degree of functional conservation across species as both the human and budding yeast orthologues were able to replace fission yeast GCPs Alp4 and Alp6 in forming the γ-tubulin complex (Riehlman et al., 2013).

In contrast to budding yeast, most other eukaryotes possess three additional members of the family, GCP4, GCP5 and GCP6, marked by the presence of the characteristic grip1 and grip2 motifs (Gunawardane et al., 2000; Murphy et al., 2001; Guillet et al., 2011; Teixidó-Travesa et al., 2012). Even though the additional GCPs are required to assemble γTuRC, they are not essential for microtubule assembly and viability in organisms such as fission yeast or *Drosophila* (Anders et al., 2006; Vérollet et al., 2006; Xiong & Oakley, 2009). On the other hand, in human cells knockdown of GCP4, GCP5 or GCP6 impairs γ-tubulin recruitment to the centrosome and mitotic spindle assembly suggesting a requirement for the complete γTuRC (Bahtz et al., 2012; Cota et al., 2017).

Additional non-GCP proteins such as NEDD1/GCP-WD (Gunawardane et al., 2003; Haren et al., 2006; Lüders et al., 2006), MZT1 and MZT2 (Hutchins et al., 2010; Teixidó-Travesa et al., 2010; Janski et al., 2012; M. Nakamura et al., 2012; Dhani et al., 2013; Masuda et al., 2013; Cota et al., 2017) are found to be functionally associated with γTuRC in many organisms.

γTuRC assembly and structure

Earlier studies of γ-tubulin complexes described the existence of a γ-tubulin small complex (γTuSC) and a γ-tubulin ring complex (γTuRC), and suggested the small complex to be the constituent subunit of the ring complex (Moritz et al., 1998). In budding yeast, the γTuSC is present in the cytoplasm as a heterotetramer consisting of laterally associated Spc97 and Spc98 bound to one molecule of γ-tubulin each, forming a Y shaped structure (Vinh et al., 2002; Kollman et al., 2008). Observations of budding yeast microtubule minus ends being capped in a similar manner to that of γTuRC capped minus ends from other systems suggested the possibility that γTuSCs might oligomerize to form a γTuRC (Byers et al., 1978;

Keating & Borisy, 2000; Moritz et al., 2000; Wiese & Zheng, 2000). Moreover, the presence of microtubules in cells with mostly 13 protofilaments suggests a strict control of geometry by the nucleator complex (Tilney et al., 1973; Evans et al., 1985). Taken together it would suggest a requirement for the formation of a higher order oligomer of γTuSC units, to form a structurally similar unit to that of γTuRCs observed in other systems that dictates the microtubule geometry. Indeed, such a higher order oligomerization is observed *in vitro* in the presence of the budding yeast SPB bound adapter Spc110 (Kollman et al., 2010). Binding to Spc110 stabilizes the lateral interaction between γTuSC units and forms a long helical filament with a cross sectional symmetry similar to that of microtubules. The overlap between two of the γTuSC subunits upon one full turn of the helix essentially gives rise to a 13-fold γ-tubulin symmetry to potentially facilitate a 13 protofilament microtubule assembly by these structures. Given that the interaction of γTuSC with Spc110 is involved in recruiting the complex to the yeast SPB (Knop, 1997a), this potentially orchestrates a mechanism for higher order γTuSC assemblies specifically at the MTOCs (Erlemann et al., 2012) and thereby prevent ectopic microtubule nucleation.

On the other hand, the individual γTuSC units as well as the filamentous oligomers displayed spacing of the γ-tubulins that did not perfectly match microtubule symmetry, suggesting an inactive conformation. Changing this would require repositioning of the γ-tubulins, potentially induced by binding of an activator, to match the spacing of tubulin in the microtubule lattice. Indeed, neither of these complexes showed strong microtubule nucleation *in vitro* (Kollman et al., 2010).

On the contrary, in higher eukaryotes, the γTuRC is found pre-assembled as a ring in the cytoplasm. It is estimated that more than 80% of the γ-tubulin in the cells is present in the cytosol as part of a preassembled complex (Moudjou et al., 1996; Stearns & Kirschner, 1994), raising the question as to how cells prevent ectopic microtubule nucleation from non-MTOC associated γTuRC. Given the low nucleation activity of γTuRCs purified from cytoplasm (Choi et al., 2010; Thawani et al., 2018), one can reach the conclusion that the cytoplasmic pool of γTuRC is not an efficient nucleator and requires additional factors that are present

only at the MTOCs. This idea is further corroborated by insights from the recent structural studies of the complex.

Four independent CryoEM structures of γTuRC, one of *Xenopus* γTuRC and the other three of the human complex including one of reconstituted recombinant γTuRC produced in our lab, collectively describe an asymmetric architecture of the γTuRC, which is not a perfect fit to be a template for nucleating microtubules (Consolati et al., 2020; P. Liu et al., 2020; Wieczorek, Urnavicius, et al., 2020; Zimmermann et al., 2020). According to these models, γTuRC is a cone shaped structure with a helical pitch. The composition of this cone includes four γTuSC units laterally connected in a semicircular arrangement, which is then continued by γTuSC-like units composed of GCP4-GCP5 and GCP4-GCP6 pairs and ends with a final γTuSC unit that partially overlaps with the first γTuSC unit. The asymmetry in the structure is apparent after the first four γTuSCs, as the following GCP4-containing units do not maintain the circular symmetry but are splayed outwards. This confers the complex a rather elliptical shape, in contrast to the perfect circular symmetry of a microtubule cross section. Moreover, similar to the γTuSC oligomer from budding yeast (Kollman et al., 2010), the γTuRC structure also contains γ-tubulin molecules with irregular lateral spacings compared to the tubulins in a microtubule lattice (**Figure I1.6**) (Consolati et al., 2020; P. Liu et al., 2020; Wieczorek, Urnavicius, et al., 2020; Zimmermann et al., 2020). Notably, these purified γTuRCs showed only moderate microtubule nucleation activity *in vitro*.

Altogether the available structural information confirms the existence of inactive γTuRC in a so-called open configuration available in the cytoplasm of higher eukaryotes. This also implies that an additional activation step might be involved in converting these complexes into efficient nucleators, possibly through conformational changes to match the symmetry of microtubules (Kollman et al., 2015). Such a mechanism would be ideal to limit microtubule nucleation to MTOCs, where specific activating factors can be utilized to regulate nucleation activation.

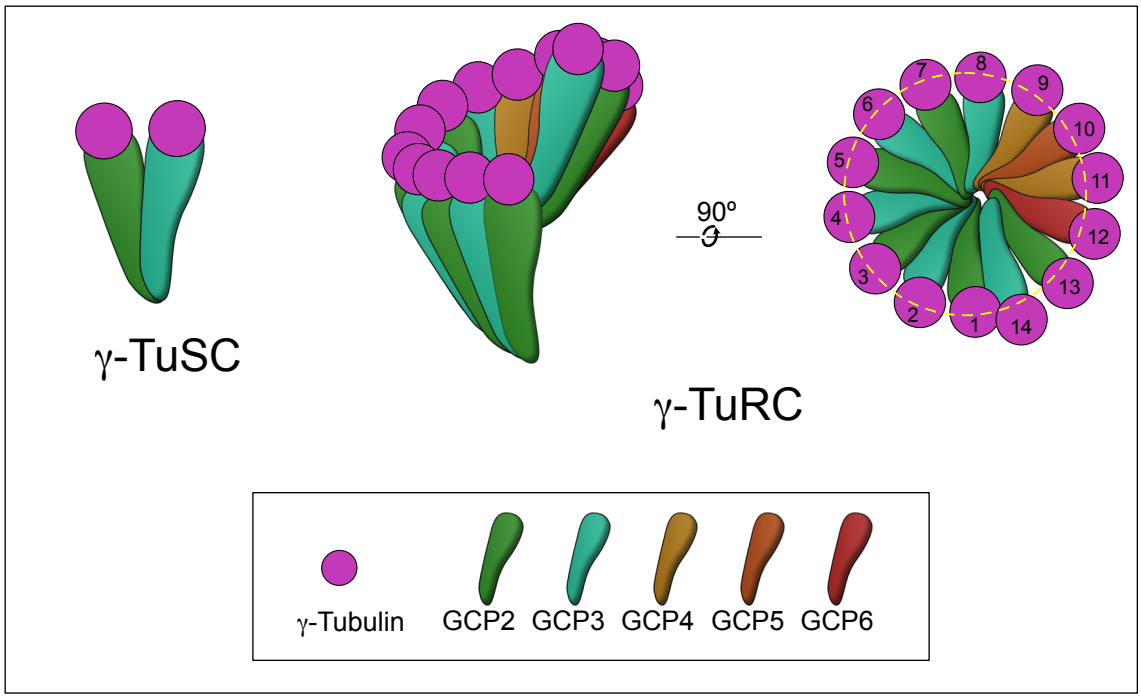


Figure I1.5: The γ-tubulin complexes

Structures of the γ-tubulin small complex (γTuSC) and the ring complex (γTuRC). The colors represent the different subunits as indicated below. The deviation from the expected circular symmetry (marked by the dashed circle) in the γ-TuRC structure can be seen in the top view (on the right).

Additional roles of γTuRC

The primary role of γTuRC is to act as a template for microtubule nucleation as described above, but γTuRC also demonstrates the ability to bind to the minus ends of pre-formed microtubules thereby stabilizing the end (Wiese & Zheng, 2000). Minus end capping function of γ-tubulin complexes, specifically of γTuSCs, independent of their roles in nucleation was described in fission yeast (Anders & Sawin, 2011). Such a function might be crucial in microtubules that are released from nucleation sites, a process commonly seen in many differentiated cell lines (Bellett et al., 2009; Moss et al., 2007; Rodionov & Borisy, 1997). Apart from ends stabilization, such a capping by γTuRC might also contribute to anchoring microtubules to the MTOCs. At the centrosome, a role of γTuRC in positioning and orienting the microtubules properly in addition to nucleation was suggested based on the observation from *C. elegans*, where γ-tubulin depletion in embryos did not completely

eliminate microtubule nucleation, but led to impaired minus end organization and centriole integrity (O'Toole et al., 2012).

A striking example of multiple functions of γTuRC is observed in keratinocytes. In these cells, γTuRC bound to CDK5RAP2 carry out the essential function of microtubule nucleation, while another distinct pool of γTuRC bound to NEDD1 is required for proper anchoring of microtubules to the cortical sites (Muroyama et al., 2016).

At the centrosome, apart from the well-established PCM localization, γTuRC is also present inside the centriolar lumen (Fuller et al., 1995; Moudjou et al., 1996; Bahtz et al., 2012; Lawo et al., 2012; Mennella et al., 2012; Sonnen et al., 2012; Schweizer et al., 2021) and at the SDAs of mature mother centrioles (A.-M. Tassin et al., 1998; Hagiwara et al., 2000; Clare et al., 2014; Q. P. H. Nguyen et al., 2020; Chong et al., 2020). Recent advances in super resolution imaging techniques such as expansion microscopy (ExM) has allowed us to observe such distinct populations of γTuRC at the centrosome, which raised the possibility that γTuRC might be involved in more functions other than microtubule nucleation. For example, the lumen-associated γTuRC is important for maintaining the structural integrity of centriolar cylinders and is believed to not directly take part in microtubule nucleation or capping functions (Schweizer et al., 2021). As the SDAs are considered to be the major microtubule anchoring sites at the centrosomes, presence of γTuRC at this location might implicate an anchoring function here, although this needs to be tested. In addition, γTuRC plays an essential role in centriole duplication, but whether this function is purely to nucleate the centriolar wall microtubules or if γTuRC has broader roles here is not fully clear (Ruiz et al., 1999; Lüders et al., 2006; Haren et al., 2006; Schweizer & Lüders, 2021). An interesting non-canonical function for γTuRC has been recently described at the ciliary basal bodies, where the complex recruits KIF2A to promote cilia disassembly (Shankar et al., 2022).

γTuRC independent microtubule nucleation

Despite the importance of γTuRC in nucleating and organizing microtubules in cells, it has been observed in several instances that cells do manage to nucleate microtubules in the absence of γTuRC components, although with much lower efficiency (Strome et al., 2001;

Hannak et al., 2002; Rogers et al., 2008; S. Wang et al., 2015; Chinen et al., 2015; Sallee et al., 2018). Some of these observations might result from the residual γTuRC components remaining in the cells after depletion.

Interestingly, the microtubule polymerase XMAP215 family protein ch-TOG and the Ran pathway target TPX2 have been shown to be able to nucleate microtubules *in vitro* without γTuRC (Roostalu et al., 2015). It is suggested that they associate with nascent microtubule intermediates to stabilize them and increase the growth rates. TPX2 has been implicated as an essential factor required for branching microtubule nucleation as well as chromatin-mediated microtubule formation in mitosis but both these processes also involve the universal nucleator γTuRC in cells (Gruss et al., 2001; Alfaro-Aco et al., 2017, 2020; Petry et al., 2013; Groen et al., 2009). γTuRC-independent *in vitro* nucleation activity was also attributed to minus end-stabilizing protein CAMSAP2, which supposedly forms phase separated condensates that promote the longitudinal interactions of tubulin dimers to form a protofilament ring, which then act as the nucleation intermediate for further microtubule growth (Imasaki et al., 2022).

A recent study that used a degron-based approach to eliminate γ-tubulin in colon cancer cells identified microtubule-associated proteins CLASP1 and TPX2 to be crucial for nucleation in the absence of γ-tubulin, while ch-TOG and CAMSAP2 also contributed to this process (Tsuchiya & Goshima, 2021). Similarly, the presence of a microtubule population at the apical non-centrosomal MTOCs in *C. elegans* intestinal epithelial cells upon removal of the γTuRC component GCP3 suggested the existence of γTuRC-independent microtubule formation, and was shown to be dependent on the XMAP215 orthologue Zyg9 (Sallee et al., 2018).

While it is possible that cells adopt γTuRC-dependent and -independent modes of microtubule nucleation, depending on the cell type or MTOCs, there is no clear consensus as to whether such distinct mechanisms work in parallel or if the observed γTuRC independent microtubules are a result of compensatory activities. Importantly, if such mechanisms are active, it raises additional questions regarding the geometry, dynamics and

nature of microtubules formed independently of γTuRC. Of specific concern here is whether the microtubules formed without γTuRC maintain a 13 protofilament architecture.

2

RECRUITMENT AND ACTIVATION OF γTuRC AT MTOCS

2.1 γTuRC recruitment to MTOCs

The primary role of γTuRC, based on our understanding so far, is to nucleate microtubules, a process that is spatially restricted to specific sites known as MTOCs and temporally regulated based on the needs pertaining to the cell type or cell cycle stages. Given that γTuRC is preassembled in the cytoplasm (Moudjou et al., 1996; Stearns & Kirschner, 1994), its recruitment to the MTOCs and its activation are two crucial processes that modulate microtubule organization in cells. The diversity among the nature and composition of MTOCs strongly suggests similar variety among the recruitment and activation factors to allow nucleation from these diverse sites.

The group of proteins characterized by the presence of an N-terminal centrosomin motif 1 (CM1) domain, including the *Drosophila* Centrosomin (Cnn) and human CDK5RAP2 have been widely associated with γTuRC recruitment function. In budding yeast, the CM1 containing protein Spc110 recruits the γTuSC to the centrosome equivalent SPB, where the complex undergoes oligomerization to become an active nucleator (Knop, 1997a, 1997b; Knop & Schiebel, 1998; T. Nguyen et al., 1998; Kollman et al., 2010, 2015; Lyon et al., 2016; T. Lin et al., 2016; Brilot et al., 2021). The closed mitosis in budding yeast is facilitated by a nuclear envelope bound SPB that organizes spatially distinct groups of microtubules on the cytoplasmic side and the nuclear side as described in the previous chapter. Interestingly, the Spc110 is localized only at the inner plaque of the SPBs (Knop, 1997a). The cytoplasmic side of the SPB utilizes a different factor, the Spc72, which similar to Spc110, contains a CM1 domain and interacts with the γ-tubulin complex components Spc97/98 to ensure the recruitment of the complex at this site for organizing the cytoplasmic microtubules (Knop & Schiebel, 1998).

In fission yeast, Spc110 orthologue Pcp1 behaves in a similar manner to target the γ-tubulin complex to the nuclear side of the SPB during mitosis (C. S. Fong et al., 2010) and the Spc72 orthologue Mto1 is involved in γTuRC function at the interphase MTOCs (Flory et al., 2002; Sawin et al., 2004; Venkatram et al., 2004; Samejima et al., 2005; S. Zimmerman & Chang, 2005; C. S. Fong et al., 2010; W. Liu et al., 2019).

Drosophila Centrosomin notably has a cell type-specific function. In proliferating cells, it is required for targeting γTuRC to the centrosome (Timothy et al., 1999; Vaizel-Ohayon & Schejter, 1999; J. Zhang & Megraw, 2007; Conduit et al., 2014; Eisman et al., 2015), but there exists a testis-specific isoform of Centrosomin in spermatid cells that recruit γTuRC to the mitochondrial surface, which is a unique MTOC found in these cell types (J. V. Chen et al., 2017; Noguchi et al., 2011). Interestingly, in *Drosophila*, in the absence of the core γTuRC components GCP4-6, centrosomal γ-tubulin recruitment and nucleation are slightly reduced but still occur, suggesting involvement of γTuSCs, possibly in oligomeric form, in which γ-tubulin is bound only to GCP2/3 similar to the situation in budding yeast (Vérollet et al., 2006; Z. Zhu et al., 2023). On the other hand, in human cells, γ-tubulin is recruited to the centrosome only as part of the whole γTuRC complex (Cota et al., 2017).

Similar to the CM1-containing proteins from organisms such as *Drosophila*, budding yeast and fission yeast, human CDK5RAP2 is also implicated in γTuRC recruitment to MTOCs. While primarily associated with tethering γTuRC to the PCM at the centrosome (K.-W. Fong et al., 2008), CDK5RAP2 has also been described to be involved in γTuRC recruitment to the Golgi membrane, a prominent non-centrosomal MTOC in some human cells (Z. Wang et al., 2010). Similar to the observations form other species, the conserved N-terminal CM1 domain mediates the interaction of human CDK5RAP2 with the γTuRC (K.-W. Fong et al., 2008; Choi et al., 2010), whereas its centrosomal and Golgi localization involves a similar sequence at the C-terminal region called the centrosomin motif 2 (CM2) (Z. Wang et al., 2010).

At the centrosome, a major pool of γTuRC is associated with PCM, hence it is no surprise that the PCM scaffold protein Pericentrin (PCNT, also known as Kendrin) and its paralogue AKAP450/AKAP9/CG-NAP are implicated in γTuRC tethering at the centrosome. Interesting to note here is that while both these proteins play an important role in γTuRC localization at the mitotic PCM, they are not crucial for γTuRC attachment to the PCM in interphase (Takahashi et al., 1999, 2002; W. C. Zimmerman et al., 2004; Gavilan et al., 2018; F. Chen et al., 2022). Homologous proteins identified in other eukaryotic organisms all characterized by a conserved pericentrin and AKAP450 centrosomal targeting (PACT)

domain in the C-terminus perform similar function in γTuRC attachment at the mitotic centrosomes/SPBs (Kawaguchi & Zheng, 2004; T. Lin et al., 2015). The PACT domain enables the exclusive centrosomal localization of these proteins (Gillingham & Munro, 2000), except in the case of AKAP450 which shows additional Golgi localization (Takahashi et al., 1999). Interesting to note here is that, PCNT and AKAP450 are related to the budding yeast Spc110, yet the human proteins do not have a functional CM1 domain. It can be speculated that either the active role of CM1 containing CDK5RAP2 in human cells, or the absence of a CM1 mediated oligomerization as the essential mechanism for γTuRC assembly as seen in budding yeast might have rendered this domain unnecessary in PCNT and AKAP450 leading to degeneration (T. Lin et al., 2015).

While PACT domain-containing proteins and CM1-containing proteins are crucial in γTuRC localization at the mitotic centrosome/SPBs, recruitment of γTuRC to interphase centrosomes seems to depend more strongly on other factors. NEDD1 plays a major role in γTuRC recruitment to the centrosome in both interphase and mitosis in human cells through direct interaction with γ-tubulin (Haren et al., 2006; Lüders et al., 2006; Manning et al., 2010). NEDD1 was first identified in *Drosophila* as a component of the γTuRC, but the lack of characteristic grip domain separated it from the members of the core GCP family (Gunawardane et al., 2003) and this separation is functionally observed as lack of NEDD1 did not prevent the assembly of a stable γTuRC, that can bind to microtubules (Lüders et al., 2006).

In addition to the well described PCM localization, γTuRC is observed to localize to specific areas of the centriolar cylinders as described in the previous chapter and many of these localizations depend on NEDD1. At the walls of centrioles, NEDD1 displays the same localization pattern as γ-tubulin, suggesting they are part of the same complex (Sonnen et al., 2012; Schweizer et al., 2021) by which NEDD1 recruits γTuRC here. Notably at this site, NEDD1 is further dependent on an additional attachment factor, CEP192, an orthologue of the *C. elegans* SPD2 (Schweizer et al., 2021; Schweizer & Lüders, 2021). In *C. elegans*, SPD2 is known for its role in in PCM recruitment and centriole biogenesis (Pelletier et al., 2004). Depletion of CEP192 in human cells eliminates both γ-tubulin and NEDD1 signals from the outer wall of the centrioles, leaving the luminal γTuRC (Schweizer et al., 2021). NEDD1

is associated with the γTuRC population in the centriole lumen as well, where its localization is mediated through interaction with the Augmin complex (Schweizer et al., 2021). In addition, both NEDD1 and γTuRC have been observed at the SDAs of the mother centrioles (Chong et al., 2020; Clare et al., 2014; Hagiwara et al., 2000; Q. P. H. Nguyen et al., 2020). The SDA protein NIN, that is also implicated in anchoring functions at centrosomal and non-centrosomal sites, has also been described to recruit γTuRC and thereby promote nucleation (S. Wang et al., 2015). Notably, the anchoring role of NIN at many of the non-centrosomal MTOCs might include γTuRC recruitment to ensure capped microtubule minus ends. However, it is not clear if NIN is directly involved in recruiting the γTuRC- NEDD1 complex to the SDAs and whether the complex performs an anchoring function at this site.

One potential candidate for γTuRC recruitment to the SDAs is the mother centriole associated Ninein-like protein (NINL) although its association with the SDAs is only a prediction based on its sequence similarity with NIN (Casenghi et al., 2003). NINL has been shown to directly interact with γTuRC and recruit it to ectopic sites, which will be discussed in detail in a later section.

Important to note here is that the luminal and centriole wall associated γTuRC populations are not known to be nucleating microtubules, and although microtubule nucleation from distal regions of the centrioles apart from PCM has recently been observed in work from our lab (Schweizer et al., 2021), it is not clear if it is mediated through the SDA associated γTuRC-NEDD1 complex. Taken along with the observation from keratinocytes that NEDD1-bound γTuRC functions as an anchoring complex rather than a nucleator (Muroyama et al., 2016), it might indicate a role of NEDD1 in the recruitment of γTuRC specifically for non-nucleating functions. Contrary to this idea is the observation from U2OS cells where NEDD1 mediated γTuRC recruitment was found to be essential for centrosomal as well as chromatin mediated nucleation (Haren et al., 2006; Lüders et al., 2006). Moreover, NEDD1 is also important for recruiting γTuRC to the lattice of existing microtubules to enable the branching microtubule nucleation, a process that also requires the Augmin complex (Goshima & Kimura, 2010; Petry et al., 2013; Alfaro-Aco et al., 2020).

On the other hand, CEP192 occupies an important position in the hierarchy of γTuRC recruitment to interphase and mitotic centrosomes. Apart from the centriole wall localization, CEP192 is important for γTuRC PCM localization as seen from the loss non-luminal populations of both γTuRC and NEDD1 upon CEP192 depletion (Schweizer et al., 2021). CEP192 is critical for assembling the PCM components in interphase and mitosis, therefore its role in γTuRC recruitment is central, although possibly indirect (Pelletier et al., 2004; Gomez-Ferreria et al., 2007; F. Zhu et al., 2008).

The γTuRC associated protein MZT1 has been implicated in the centrosomal recruitment of the complex although this might be an indirect effect resulting from it being an integral structural component of the γTuRC at least in vertebrates (T. Lin et al., 2016; Cota et al., 2017; Wieczorek, Huang, et al., 2020; Wieczorek, Urnavicius, et al., 2020; P. Liu et al., 2020; Consolati et al., 2020; Zimmermann et al., 2020). On the contrary, the fission yeast orthologue of MZT1 was found to be essential for γ-tubulin complex recruitment to the MTOCs, but not required for the assembly of the complex (Dhani et al., 2013; Masuda et al., 2013; Masuda & Toda, 2016). Interestingly, the *C. elegans* orthologue of MZT1, while necessary for γTuRC recruitment to the PCM, is dispensable for recruiting the complex to centrioles as well as apical non-centrosomal MTOCs in intestinal epithelial cells (Sallee et al., 2018). Given MZT1 colocalizes with GCP3 orthologue at the apical MTOCs in these cells under normal conditions, it could still be associated with the γTuRC complex, but it is not an essential component for neither γTuRC integrity nor recruitment at this site.

2.2 Activating nucleation from γTuRC

Despite being widely regarded as the universal nucleator, the γ-tubulin complexes are not very efficient in nucleating microtubules by themselves and require additional factors to effectively carry out this function (Moritz et al., 1998; Kollman et al., 2011; Teixidó-Travesa et al., 2012; Roostalu & Surrey, 2017; Consolati et al., 2020; Oegema et al., 1999). The soluble cytoplasmic version of γTuRC may be considered its inactive form and recent studies provided crucial information indicating that the asymmetric structure of γTuRC does not match the microtubule structure very well, making it an imperfect template for nucleation (Consolati et al., 2020; P. Liu et al., 2020; Wieczorek, Urnavicius, et al., 2020; Zimmermann et al., 2020). While this asymmetry may prevent ectopic nucleation, it also suggests a need for specific activation of γTuRC at MTOCs with the help of additional activating factors.

One immediate question derived from the structural data is whether the γTuRC ring undergoes a conformational change to be an active nucleator at the MTOCs and if so, what key players contribute to such a process. Cryo-EM tomography of budding yeast SPBs indicate that microtubule minus ends *in vivo* are attached to the SPBs vis a γTuSC ring-like oligomer with a helical pitch lower than the *in vitro* reconstituted γTuSC filaments, but similar to that of a microtubule (Kollman et al., 2010, 2015). On the other hand, disulphide crosslinking to trap the γTuSC oligomer in such a compact or 'closed' conformation increased its *in vitro* nucleation ability only up to 2-fold. While this may support 'ring closure' as a possible mechanism for converting γ-tubulin complexes into an active nucleator, additional activating factors are likely necessary for achieving the levels of microtubule nucleation seen in cells. Hence, so far it is not clear if structural rearrangements leading to a closed conformation of γTuRC (or of a γTuSC oligomer) are an essential universal prerequisite to stimulate nucleation or occur subsequently as a consequence of having microtubules bound to the nucleator. Notably, certain centrosome-associated proteins have been observed to enhance the nucleation ability from purified γTuRCs *in vitro*, such as the CM1 domain-containing γTuRC binding proteins and the TOG domain-containing microtubule polymerases.

CM1 domain containing proteins as γTuRC activators

In budding yeast, the CM1 protein Spc110 is widely regarded as an activator of nucleation owing to its role in SPB recruitment of the γTuSC and the ability to induce γTuSC oligomerization as described in the previous sections. But such an oligomer displayed wider γ-tubulin spacings, making it a so called 'open' configuration with considerably low nucleation potential *in vitro*, which invites doubts on whether the proposed CM1 domain binding mediated activation of γ-tubulin complex is really the case (Kollman et al., 2010; Vinh et al., 2002). Studies based on γ-tubulin complex from *Candida albicans* identified a role for MZT1 in addition to Spc110 in the formation of an active oligomeric nucleation template (T. Lin et al., 2016). Although budding yeast lacks a MZT1 gene, it cannot be ruled out that while Spc110 binding is necessary for γTuSC recruitment to the SPBs, additional elements at the SPB may contribute to activating nucleation from the oligomeric γ-tubulin complex.

A direct role of CM1 binding-mediated γTuRC activation was demonstrated by an increased nucleation ability from purified human γTuRC *in vitro* upon incubation with either the full length CDK5RAP2 protein or just the CM1 containing region (amino acids 51-100) (Choi et al., 2010). The authors refer to this short stretch of amino acids as the γTuRC-mediated nucleation activator or γTuNA. A point mutation that disrupts the binding of γTuNA (and the full length CDK5RAP2) to the γTuRC did not show such an effect, further confirming the need for CM1 binding in nucleation activation. By contrast, recently reported *in vitro* assays with purified *Xenopus* γTuRC failed to produce any such increase in nucleation even in the presence of excess of CM1 (P. Liu et al., 2020; Thawani et al., 2020). Interestingly, a subsequent study observed an enhancement of nucleation from *Xenopus* γTuRC *in vitro* in the presence of CM1 domain purified with Strep-His tag, while the presence of a Halo tag inhibited such an action (Rale et al., 2022). In addition, this study also identified a need for dimerized CM1 for proper γTuRC activation. It is possible that the presence of bulkier tags used in purification might hinder either CM1 dimerization or its activity somehow, but conflicting to this idea is the earlier reports from the same lab where CM1 with a similar smaller tag did not produce much effect on *in vitro* γTuRC activation (Thawani et al., 2020). Adding to this is the observation that human γTuRC purified with CM1 domain bound to it

still appeared to have an open asymmetric, likely inactive conformation (Wieczorek, Urnavicius, et al., 2020).

Interesting to note here is the observation that overexpression of just the CM1 containing 51-100 amino acid region of human CDK5RAP2 in cells induces microtubule nucleation throughout the cytoplasm suggesting that the presumably inactive γTuRC pool in the cytosol is somehow activated by the binding of CM1 domain. Unlike the *in vitro* nucleation activation, this cellular effect has been consistently reproduced by others and in our own experiments. Moreover, knock-down of the endogenous CDK5RAP2 did not affect this ectopic nucleation by CM1 overexpression. Whether binding of CM1 is sufficient for activation in this system or additional factors are involved, has not been carefully tested.

Other putative activating factors include TPX2 (Roostalu et al., 2015), an essential factor in branching microtubule nucleation that contains a partial CM1 like region (Alfaro-Aco et al., 2017; R. Zhang et al., 2017; Tovey & Conduit, 2018) and NME7, a member of the NME kinase family that is found to be associated with γTuRC (Teixidó-Travesa et al., 2010; Hutchins et al., 2010; Choi et al., 2010; P. Liu et al., 2014). When incubated with purified *Xenopus* γTuRC, both TPX2 and CM1 demonstrated mild increase in nucleation by less than 1.5 fold (Thawani et al., 2020). Similarly, the effect of NME7 on γTuRC mediated nucleation was very mild or negligible based on independent studies (P. Liu et al., 2014; Thawani et al., 2020). On the other hand, the TOG domain containing microtubule polymerase XMAP215 showed a substantial increase in nucleation ability up to 25-fold under the same experimental conditions (Thawani et al., 2020).

In summary, binding of CM1 domain from CDK5RAP2 might be a step in converting γTuRC into an active nucleator, however, more information regarding what additional factors and mechanisms contribute to such an activation remains to be unraveled and the question as to whether this is a prominent mechanism of microtubule nucleation in cells needs to be addressed.

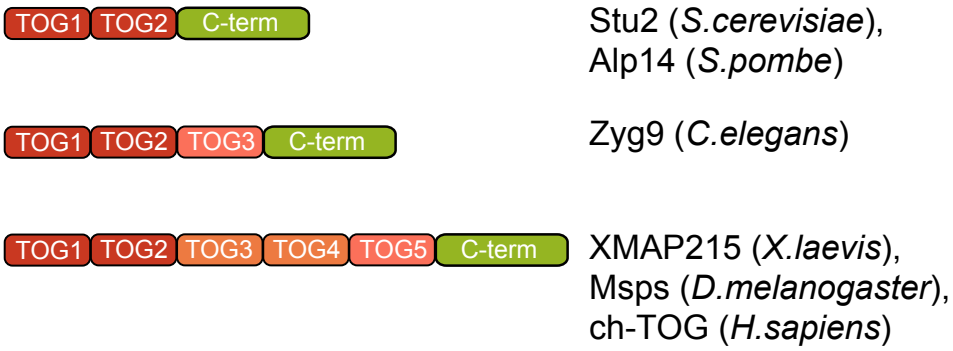
TOG domain containing proteins as microtubule nucleators

Members of the XMAP215 family have been identified in a variety of organisms as polymerases that catalyze the growth of microtubule plus ends (Gard & Kirschner, 1987; Gard et al., 2004; Brouhard et al., 2008; Al-Bassam et al., 2012; W. Li et al., 2012; Podolski et al., 2014). One defining feature of this group of proteins is the presence of tubulin binding TOG domains that vary from two to five between different species and have microtubule associated functions, notably in relation to spindle dynamics (Gard et al., 2004). As a plus ends associated microtubule regulator, they are also involved in kinetochore-microtubule attachments (K. Tanaka et al., 2005; M. P. Miller et al., 2016).

The differential ability of the TOG domains to bind to lattice bound vs soluble tubulin dimers is key to their role in plus end growth (Brouhard et al., 2008; Widlund et al., 2011; Roostalu & Surrey, 2017). Each TOG domain consists of six HEAT repeat motifs that provide the ability to bind tubulin (Al-Bassam et al., 2007; Slep & Vale, 2007). The budding yeast orthologue Stu2 and the fission yeast protein Alp14 carry two TOG domains each but their ability to homodimerize essentially provide four functional TOG domains (Al-Bassam et al., 2006, 2012; Brouhard & Rice, 2014; Podolski et al., 2014; Nithianantham et al., 2018). The *C. elegans* Zyg9 is the only observed orthologue with three TOG domains, whereas orthologs of higher eukaryotes display an array of five TOG domains and are known to function as monomers (Brouhard et al., 2008; Al-Bassam & Chang, 2011).

Importantly, the TOG domains themselves display differences in structure as well as their ability to bind to tubulin dimers. Based on studies from yeast proteins, the TOG1 and TOG2 domains typically have very similar structure that facilitates the binding to the curved surface of free α/β-tubulin dimers (Al-Bassam et al., 2006; Slep & Vale, 2007; Ayaz et al., 2012). Structure of TOG domain 3 as obtained from the *Drosophila* homologue Msps revealed a slightly different architecture compared to TOG1&2, yet contained a similar tubulin binding surface (Howard et al., 2015), whereas TOG4 demonstrated a very distinct architecture than the others suggesting a different mode of tubulin binding (Fox et al., 2014). It is predicted that the presence of four TOG domains in higher eukaryotes eliminates the need for dimerization as seen in the yeast proteins Stu2 and Alp14. By contrast the fifth TOG domain presents a very unique structure. Although quite divergent from the rest of the TOG

a.



b.

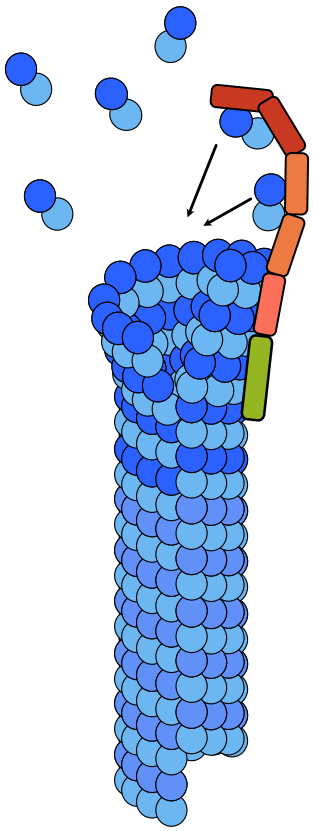


Figure I2.1: Microtubule polymerases of the XMAP215 family

a. Domain organization of the members of the XMAp215 family. The similar colors indicate the degree of sequence conservation between the domains. The names of the individual members indicated on the right side with the corresponding organism in the brackets.
b. Scheme of polymerase activity by an XMAP215 family member with pentameric TOG array.

domains, the TOG5 present in proteins with a pentameric TOG array is very similar to the TOG3 from *C. elegans* Zyg9 (**Figure I2.1a**) (Al-Bassam et al., 2007; Al-Bassam & Chang, 2011; Byrnes & Slep, 2017). The structure of TOG5 from the *Drosophila* Msps and that of TOG3 present in Zyg9, both reveal the presence of an additional HEAT repeat element at the N-terminus that is orthogonal to the typical six HEAT repeats that all TOG domains share (Al-Bassam et al., 2007; Byrnes & Slep, 2017). Interestingly this extra HEAT repeat confers the protein the ability to bind specifically to lattice bound tubulin dimers, in contrast with the specific affinity of other TOG domains towards free soluble tubulin dimers. More importantly, the order of these TOG domains needs to be strictly maintained for optimal polymerization activity (Byrnes & Slep, 2017). This implies a mode of action by which lattice bound XMAP215 family proteins use their N-terminal TOG domains to bind to soluble tubulin and incorporate it into the growing ends while TOG5 and other C-terminal lattice binding elements facilitate the progressive movement of XAMP215 along the lattice, following the growing plus end (**Figure I2.1b**).

The role of XMAP215 family proteins in nucleation in addition to plus end growth was demonstrated by several *in vitro* studies. Xenopus egg extract depleted of XMA215 behaved similar to that of γ-tubulin depleted extracts when tested for the ability to assemble microtubules around chromatin coated beads (Groen et al., 2009). Interestingly, supplying with excess of XMAP215 was able to partially rescue the effect of γ-tubulin depletion in this experiment, further suggesting an intrinsic ability of XMAP215 proteins to nucleate microtubules. While it has been shown that XMAP215 family proteins can enhance the spontaneous microtubule nucleation *in vitro* (Roostalu et al., 2015), they also function along with γTuRC *in vitro* to facilitate templated nucleation (Wieczorek et al., 2015).

The Xenopus XMAP215 has been shown to be necessary to confer nucleation ability to salt-stripped centrosomes (Popov et al., 2002) and apart from that, they are also able to stimulate nucleation from purified, surface bound γTuRC molecules (Thawani et al., 2018; Consolati et al., 2020). Moreover, the Xenopus protein has been shown to interact with γ-tubulin via its C-terminus region, which is essential for the nucleation activity (Thawani et al., 2018). The budding yeast Stu2 associates with Spc72 to promote γTuSC oligomerization and microtubule nucleation (Gunzelmann et al., 2018). Similarly, the fission yeast protein Alp14

has also been described to interact with γ-tubulin complex and stimulate nucleation *in vivo*, although a fraction of Alp14 only transiently associated with the nucleation site (Flor-Parra et al., 2018). By contrast, the human orthologue ch-TOG did not show any association with purified γTuRC (Choi et al., 2010; Hutchins et al., 2010; Teixidó-Travesa et al., 2010).

In our lab we examined the role of XMAP215 family protein ch-TOG in microtubule nucleation in human cells. A post-doctoral fellow from the lab Aamir Ali, identified that ch-TOG transiently localizes to the centrosome in a microtubule dependent manner and the presence of ch-TOG is essential for γTuRC recruitment and microtubule nucleation at the centrosome in interphase (Ali et al., 2023). A part of my thesis explores the relationship between ch-TOG and the nucleator γTuRC in human cells and its contribution towards microtubule nucleation.

NINL in activating γTuRC mediated nucleation from ectopic MTOCs

Ninein-like protein (NINL/Nlp) is a 156 KDa centrosomal protein that can bind to and recruit γTuRC as mentioned earlier. NINL was first identified as an interactor of mitotic kinase Plk1. The N-terminal half of NINL shows 37% sequence similarity with the corresponding region of the SDA protein NIN, hence the name. The sequence similarity is also reflected in the structure as both proteins contain multiple EF hand domains, known for their role in Ca^{2+} binding. In addition, NINL, similar to NIN, consist of several coiled-coil domains particularly in the C-terminal half of the protein (Casenghi et al., 2003; Redwine et al., 2017).

NINL is recruited to the centrosome by its association with the dynein-dynactin motor complex through the dynactin subunit $p150^{Glued}$, during the interphase, where it is thought to bind to γTuRC. As the cell enters mitosis, Plk1 mediated phosphorylation of NINL inhibits its interaction with γTuRC and other possible binding factors at the centrosome, leading to its disassociation from the maturing centrosome. In addition, the phosphorylated NINL no longer binds to the dynein-dynactin complex, preventing its centrosomal recruitment, and instead gets distributed in the cytoplasm until the end of mitosis (Casenghi et al., 2005). In addition to Plk1, NINL is a substrate of several mitotic kinases including

Nek2, Cdc2 and Aurora B (Rapley et al., 2005; Yan et al., 2010; X. Zhao et al., 2010). Such a strong regulation through multiple mediators indicate that the removal of NINL is perhaps an essential step in centrosome maturation and mitotic spindle assembly.

In line with that, overexpression of NINL in cells is associated with aberrant mitotic spindles, and this effect was enhanced when a phosphorylation-deficient mutant was used (Casenghi et al., 2003). Upon overexpression in human cell lines, NINL forms large assemblies around the centrosome that recruit γTuRC as seen by staining with both γ-tubulin and GCP4 and can nucleate microtubules upon regrowth (Casenghi et al., 2003). Such an accumulation of γTuRC into a larger assembly would interfere with regular spindle assembly, which might result in the observed spindle defects, but whether the persistence of endogenous NINL during mitosis would lead to a similar effect has not been verified. Consequently, NINL is found to be overexpressed in several cancer cell lines including breast and lung carcinomas and has been shown to induce tumorigenesis in mice upon over expression (Qu et al., 2008; Jin et al., 2009; Zhan, 2009; Shao et al., 2010; J. Li & Zhan, 2011; W. Zhao et al., 2012).

Interesting to note here is the ability of NNL to recruit γTuRC into larger assemblies outside the centrosome that exhibit nucleation abilities. Curiously, over expression of only the N-terminal half of the protein result in the formation of similar assemblies, but smaller and distributed throughout the cytoplasm, as opposed to a single large one at the centrosome, as obtained with full length NINL (Casenghi et al., 2003). Each of these smaller droplet-like assemblies of NINL successfully recruit γTuRC and nucleate microtubules in a regrowth assay. On the other hand, the C-terminal half upon overexpression forms larger aggregate like structures that fail to recruit γTuRC components (Casenghi et al., 2003). These results establish NINL to be a potential nucleation factor that can recruit γTuRC though its N-terminal half to create ectopic MTOCs in the cells.

In our lab we attempted to dissect the mechanism of NINL-mediated ectopic MTOC formation. A previous student from the lab, Joel Paz, examined the contribution of different regions and domains within the protein in the recruitment of γTuRC and in microtubule nucleation by testing their ability to form ectopic MTOCs. For this purpose, he generated truncated versions of NINL and targeted them to the mitochondrial surface using a

mitochondrial targeting sequence cloned from the *Drosophila* testis specific isoform of the Cnn protein (CnnT) (J. V. Chen et al., 2017). As the mitochondrial surface is not a canonical MTOC in human cells, this provided the unique opportunity to understand the minimal components needed to recruit γTuRC and stimulate microtubule nucleation.

As observed in the cytoplasmic overexpression studies, the N-terminal half of NINL (amino acids 1-702), upon targeting to the mitochondria, was able to recruit γTuRC to this site and demonstrated microtubule formation in a regrowth assay. Further dissection of the protein domains revealed two shorter regions within the N-terminus that are capable of γTuRC recruitment. A construct containing 1-442 amino acids of NINL generated a successful ectopic MTOC at the mitochondria revealing it to be the minimal region required for this process. On the other hand, an even smaller fragment of 1-287 amino acids managed to recruit γTuRC, but failed to activate nucleation upon regrowth (**Figure I2.2**). This striking result suggests, as described in the previous sections, that γTuRC requires some sort of activation to become an efficient nucleating factor.

This led us to wonder how NINL 1-442 contributes to microtubule nucleation apart from recruiting the γTuRC. It might be speculated that the presence of an additional coiled-coil domain in the NINL 1-442 region but not in the NINL 1-287 somehow activates γTuRC, making it better suited to act as a nucleation template. On the other hand, activation of nucleation may involve additional components that get recruited to the mitochondrial surface by interaction with this extra coiled-coil domain.

While NINL displays striking ability to bind to γTuRC in a Plk1 dependent manner and induce nucleation at ectopic sites, whether it is involved in microtubule nucleation from the centrosome is not very clear. One earlier study has observed a reduction in microtubule regrowth from the centrosome upon antibody-injected inhibition NINL activity (Casenghi et al., 2003). On the other hand, it is counterintuitive as to why a protein involved in microtubule nucleation is actively removed from the centrosome during mitosis, a stage that requires elevated levels of microtubule formation. Therefore, a part of this project is an attempt to address some of the questions regarding the role of NINL in microtubule assembly at the interphase centrosomes and in the formation of ectopic MTOCs.

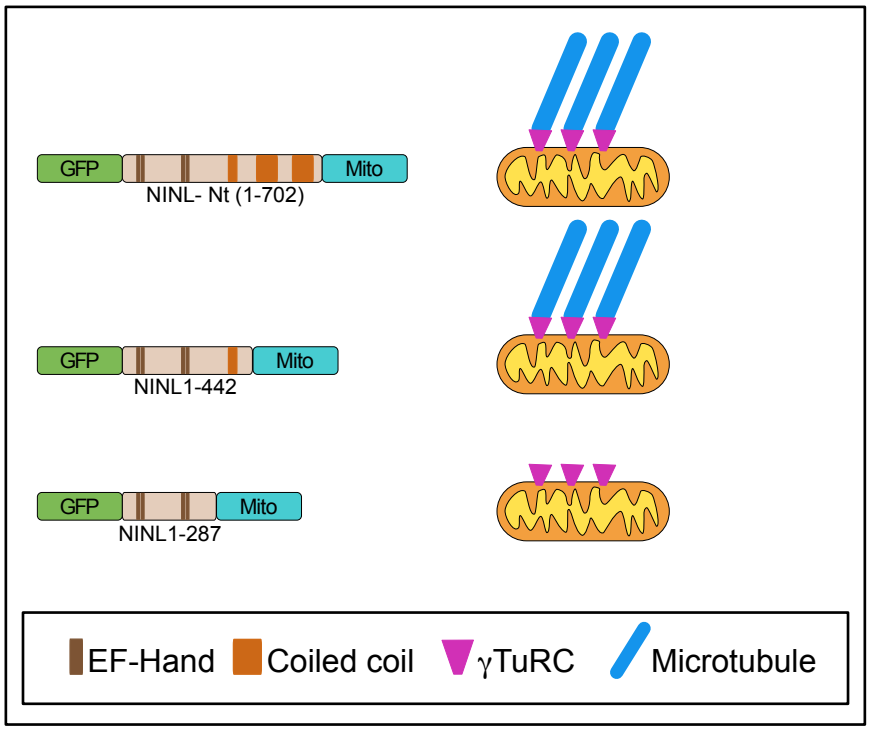


Figure I2.2: generation of ectopic MTOC by NINL

NINL N-terminus when targeted to mitochondrial surface generates ectopic MTOCs. The ability to recruit γTuRC and nucleate microtubules by smaller fragments of NINL is summarized in the figure.

Mechanisms of γTuRC recruitment and microtubule nucleation in cells

As discussed above, a wide range of factors have been implicated with γTuRC recruitment and microtubule nucleation in different scenarios. Yet, there is still no clear answer as to whether there exists a central mechanism for activating γTuRC at MTOCs or whether this involves multiple mechanisms. In this thesis I focused on three potential factors for their role in γTuRC recruitment and microtubule nucleation at centrosomal and non-centrosomal MTOCs. Firstly, I characterized NINL with regards to its role in nucleation at the centrosome and ectopic MTOCs. Secondly, I addressed how human ch-TOG influences γTuRC mediated nucleation at the centrosome and Golgi. And a third chapter aims to understand if γTuRC association with CM1 containing proteins is relevant for centrosomal microtubule nucleation.

OBJECTIVES

1. To characterize NINL at the centrosome and study its role in microtubule nucleation

2. To understand the role of ch-TOG in microtubule nucleation at centrosomal and non-centrosomal MTOCs

3. To study the relevance of γTuRC-CM1 association in centrosomal γTuRC recruitment and activation of microtubule nucleation

MATERIALS AND METHODS

Recombinant DNA production and molecular cloning

The GFP-NINL-Nt plasmid obtained from the lab of Dr. Erich Nigg was used to generate smaller N-terminal constructs of NINL with mito-targeting sequence. These were generated by a previous student in our group, Joel Paz. Firstly, the mito-targeting sequence from *Drosophila* CnnT, the testis specific isoform of Centrosomin (Cnn), obtained from Drosophila Genomics Resource Center (AT09084) was cloned into the vector pEGFP-C1 (Addgene), to generate the GFP-Mito construct used as the negative control in our experiments. Later, regions 1-287, 1-442 and 1-702 from GFP-NINL-Nt was similarly cloned into the GFP-Mito construct.

The preparations involved PCR amplification of the regions from respective sources using Phusion® high-fidelity DNA polymerase and primers that added the restriction desired restriction sites on either ends of the amplified product to generate the insert. Restriction sites used to clone Mito targeting region to pEGFP-C1 were SacII and BamHI at the 5' and 3' ends respectively. The use of SalI and SacII enzymes to clone NINL regions placed these sequences between the GFP and Mito targeting region. The vector was linearized by restriction digestion with appropriate enzymes for 2hrs at 37ºC followed by dephosphorylation by Calf Intestinal Alkaline Phosphatase (CIP) for 30 minutes.

Digested vector and amplified insert were run in a 1% agarose gel and bands were isolated by cutting the specific gel region to be purify the DNA using Nucleospin® Gel and PCR cleanup protocol. Inserts and Vectors with overlapping regions marked by the restriction sites were allowed to integrate by homologous recombination through Gibson Assembly®. NEB® 10-beta Competent E. coli (High Efficiency) bacteria were transformed with the recombinant plasmid to allow ligation and amplification. Upon colony formation in LB agar plates containing antibiotic (Kanamycin 50 μg/mL), multiple colonies were tested for the uptake of recombinant construct by isolating the plasmids from a primary overnight culture and checking by restriction digestion to find the insert. Further verification of cloning was made by performing Sanger sequencing through Macrogen sequencing service.

The BirA tagged GCP2 and GCP3 constructs were similarly prepared in the lab by Cristina Lacasa and Joel Paz using the vectors pcDNA5 FRT/TO FlagBirA* C-ter and pcDNA5 FRT/TO FlagBirA* N-ter respectively.

For experiments involving GFP-CM1 or FLAG-CM1 overexpression, constructs that has the region corresponding to amino acids 58-90 from CDK5RAP2 gene cloned into the vectors pEGFP-C1 (Cota et al., 2017) or pFLAG-CMV2 (K.-W. Fong et al., 2008) respectively were used.

Cell culture

The following human cell lines were used for the experiments in this thesis: U2OS (derived from osteosarcoma), HEK293T (immortalized embryonic kidney cells that are modified to enhance transfection and viral packaging abilities) and hTERT-RPE1 (immortalized retinal pigment epithelial cells). U2OS and HEK293T cells were cultured using Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and antibiotic solution of penicillin/streptomycin (100 IU/mL and 100 μg/mL, respectively). hTERT-RPE1 (hereafter referred to as RPE1) cells and clonal lines derived from them were grown in DMEM-F12 medium, also supplemented with FBS and antibiotics as described above. All cells were maintained in 37ºC with 5% CO2 in a humidified incubator. For experiments involving immunofluorescence, cells were grown on glass coverslips coated with poly-L-lysine in six-well plates.

Cell culture treatments

Plasmid transfection for over expression experiments

Transfection in HEK29T cells were mediated by linear polyethylenimine (PEI 25K™, Polysciences). For co-IP and BioID pull downs, HEK293T cells were grown in 15cm dishes to obtain sufficient protein content in the lysate. For transfection, 20 μg of plasmid DNA for one 15 cm dish was diluted in 2mL of OPTIMEM along with PEI to achieve a final concentration of 8 μg/mL. After incubation at room temperature for 5 minutes, the mix was added to the cells and subjected to a media change after 5 hrs.

Transfection in U2OS cells were carried out using Lipofectamine® 2000 (Invitrogen). For one well of a six well plate, 2 μg of plasmid DNA was diluted in 250 μL of OPTIMEM and in a separate tube, 7.5 μL of Lipofectamine® 2000 was diluted in 250 μL OPTIMEM. After 5 minutes of incubation at room temperature, both solutions were combined and further incubated for 10 minutes at room temperature. Following, the DNA-lipid mix was added to the cells and subjected to a media change after 5 hrs. To transfect RPE1 cells in six-well plates, 2 μg of plasmid DNA and 2 μL of TransfeX™ (ATCC®ACS-4005) reagent were mixed in 100 μL of OPTIMEM and added to the cells after 5 minutes of incubation.

Cells were collected after 48 hrs of transfection except in experiments involving mitochondrial targeting constructs or GFP-CM1 overexpression in ch-TOG depleted cells, where cells were collected 24 hrs post transfection.

siRNA treatments for gene knock-down

U2OS or RPE1 cells were treated with specific siRNAs using Lipofectamine® RNAiMAX reagent (invitrogen). For one well of a six-well plate, 5 uL of siRNA from a stock of 20 μM was diluted in 250 μL of OPTIMEM and 7 μL of Lipofectamine® RNAiMAX was similarly diluted in 250 μL OPTIMEM in a separate tube. After 5 minutes of incubation at room temperature, the solutions were combined and then incubated for another 10 minutes. Before adding the mix to the cells, the culture media was replaced by 500 μL of the reduced serum OPTIMEM, and upon adding the siRNA-Lipid mix, a final volume of 1 mL of OPTIMEM and 100 nM concentration of siRNA was achieved. The media was replaced after 5 hrs using full serum containing media and 24 hrs post siRNA treatment, cells were split once to achieve better confluency. In the case of imaging experiments, cells were grown on poly-L-lysine coated coverslips after splitting. 72 hrs after siRNA treatment, cells were collected for lysis or fixation. The siRNAs used are described in the Table M1.

Table M1: List of siRNAs used in this study

siRNA	Sequence	Supplier
Luciferase (Control)	CGUACGCGGAAUACUUCGA	Sigma
NINL oligo combination #1	GAACUACAAGGAUCAAUUA CAAAGUGAGUCUUGAGGAA CUAAAGAAGCUCAGAAUGA GACCAUUUCGCCAGGGUUA	Dharmacon (SO-3010833G)
NINL oligo combination #2	CGACCAUUUCGCCAGGGUU(dT)(dT) GCAAGGCUUGGUCUCAUUA(dT)(dT) CAGUGAGUAUAGAAACGGA(dT)(dT)	Ambion (125607) Ambion (125608) Ambion (s22765)
ch-TOG	GAGCCCAGAGUGGUCCAAA(dT)(dT)	Sigma
CEP128	GGAGCUAUCUCGAAGGUUA(dT)(dT)	Sigma

Regrowth assay

Microtubule nucleation under different scenarios were tested using a regrowth assay. Cells grown on coverslips were subjected to microtubule depolymerization by cold treatment for 30 minutes by placing the culture dish on ice or by treating with nocodazole at a final concentration of 1.6 μg/mL for 2 hrs. In the experiments that involved nocodazole treatment, the drug was washed out prior to regrowth using ice-cold PBS three times, each wash involving 5 minutes incubation on ice. Cells were then incubated with cold DMEM on ice for another 30 minutes to ensure complete removal of the drug before proceeding to the regrowth.

After depolymerization through either means, the cover slips were immersed for the specified times in DMEM media pre-warmed and maintained at 37°C in a water bath and then immediately fixed. The fixation involved either the incubation in cold methanol for 10 minutes at -20°C, or incubation at room temperature for 3 minutes with a mixture containing

2% formaldehyde in PHEM buffer (60 mM PIPES, 25 mM HEPES pH 7.4, 5 mM EGTA, 1 mM $MgCl_2$) and 0.1% Triton X-100, followed by methanol fixation at -20ºC.

The use of formaldehyde-PHEM-TritonX mixture improves the staining of microtubule with less background, but reduces the staining efficiency of centrosomal proteins, particularly of γ-tubulin. The combination of both allows better visualization of both microtubules and centrosome. But in scenarios where microtubules were not stained, only methanol fixation was performed.

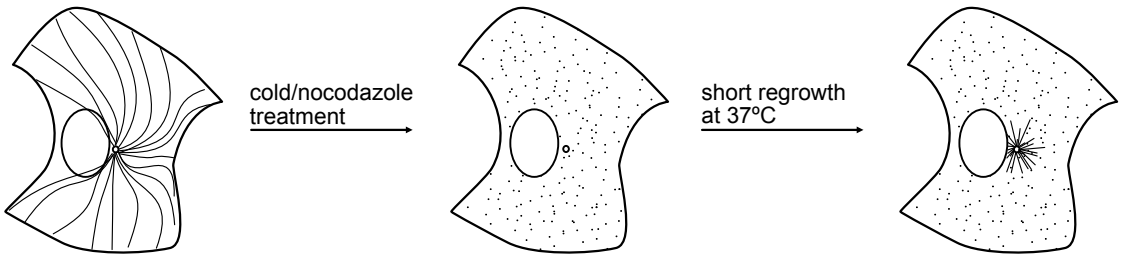


Figure M1: Schematic representation of a regrowth assay in cells.

Upon cold or nocodazole treatment, microtubules undergo depolymerization and when transferred to 37ºC, microtubules are re-formed at the MTOCs.

Immunostaining

For fluorescence-based imaging, cells grown on coverslips were fixed in either cold methanol at -20ºC or by a combination of formaldehyde-PHEM-TritonX followed by methanol. The fixed coverslips were subjected blocking using PBS-BT solution (3% BSA and 0.1% TritonX in PBS, supplemented with 0.02% sodium azide) at room temperature for 20 minutes. Following blocking, the coverslips were incubated with primary diluted in PBS-BT solution for 30 minutes, washed three times with the blocking solution and incubated with secondary antibodies for another 30 minutes. After washing the secondaries in a similar manner, cells were treated with DAPI for staining the DNA for 1 minute and washed twice with PBS. Coverslips were then mounted onto a glass slide over a drop of ProLong® Gold Antifade Reagent mounting media (Thermo Fisher) to retain strong signals.

This protocol was slightly modified when staining for ch-TOG were the primary antibody for ch-TOG was incubated overnight at 4ºC, followed by 30 minutes incubation of other primary antibodies to maximize the signal in 3D-SIM.

In the experiment involving the triple labelling of Golgi, ch-TOG and α-tubulin, the Golgi marker GM130 conjugated with Alexa fluor 647 was used, which eliminated the need for a secondary antibody. Moreover, this antibody was treated only at the very end after washing out the other primary and secondary antibodies used in this experiment to avoid any cross reactions.

Microscopy

Regular images that does not involve super/resolution, were captured using a DMI6000B microscope (Leica) with 100X or 63X oil immersion objectives having a numerical aperture (NA) 1.4. Images acquired with constant exposure settings were analyzed using Fiji (ImageJ) software. The displayed figures represent single Z-plane image.

The super-resolution images were acquired in Elyra PS.1 (Carl Zeiss, Germany) using three-dimensional structured illumination microscopy (3D-SIM) settings. The images were captured using Alpha Plan Apochromat 100x/1.46NA Oil Dic M27 objective lens with immersol 518 F oil (Zeiss). Lasers with wavelengths of 488 nm (25% of 200 mW laser source), 561 nm (20% of 200 mW laser source) and 642 nm (7% of 500 mW laser source) were used to excite the fluorescent dyes Alexa fluor 488, 568 and 647 respectively. Emission filters of 495-575 nm, 570-650 nm, and above 655 nm were used for the respective channels. An area of 256x256 pixels was used to image centrosome, 512x512 for mitotic cells and 1024x1024 for imaging an entire interphase cell. ZEISS Zen black software was used to capture the images which was then subjected to SIM processing with Manual settings involving the parameters Max.Isotrop and baseline shifted. The processed images were then analyzed using Fiji (ImageJ). The displayed figures represent the maximum intensity projection of the acquired Z-planes.

The pseudo colors cyan and Magenta represent the 488nm and 568nm channels respectively in all figures. The pseudo color yellow was used to represent either DAPI (461 nm) or far-red (647nm) channels depending on the experiment.

Quantifications and statistics

Intensity quantifications from images with multiple Z-planes were carried out on a sum-projection. For centrosomal quantifications mean intensities from a region of interest (ROI) of 1 μm X 1 μm was used and 2 μm X 2 μm ROI for mitotic poles and centrosomal asters. Following intensity measurement, background subtraction was done by using the intensity of the same ROI from an adjacent region in the same image. In **Figure R2.3**, a freeform ROI was used to measure the luminal intensity which was then subtracted from a square ROI intensity of the centrosome, to obtain PCM specific intensity. All intensity measurements were normalized to the average of the control in respective experiments and the normalized values were used to plot the graph and perform statistics.

In experiments involving microtubule counts, the absolute numbers were used in graphs and statistics. To analyze cytoplasmic nucleation upon GCP-CM1 overexpression, the number of microtubules within a ROI of 10 μm X 10 μM was counted. The ROI was chosen towards the cell periphery to ensure centrosomal and Golgi derived microtubules are not included in the counting. To quantify Golgi regrowth, microtubules associated with Golgi based on GM130 staining were counted.

Statistical analyses were performed using GraphPad Prism 9.4.0 software. The normalized intensities or absolute counts were combined from multiple replicates as indicated in the figure legends and displayed in a scatter plot with median and interquartile ranges marked. Statistical significance was determined by unpaired, two-tailed t-test with Welch's correction, performed on the averages from each trial. Additional details are found in the figure legends.

Western blot

Protein levels in cells upon siRNA mediated depletion was tested by running a western blot. Cells were lysed using lysis buffer (50 mM HEPES, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM

EGTA, 0.5% Triton X-100) supplemented with 1X Protease inhibitor (Complete™, EDTA-free Protease Inhibitor Cocktail) for 15 minutes at 4ºC, followed by centrifugation at 16000g for 15 minutes at 4ºC. The supernatant was collected and the total protein concentration in the lysate was measured using Bradford assay with BSA as standard. The lysate was mixed with 6X sample buffer (83 mM Bis-Tris, 50 mM HCl, 3.3% glycerol, 1.3% SDS, 0.3 mM EDTA, 0.01% bromophenol blue, 0.83% β-mercaptoethanol) such that the final concentration of loading buffer is 1X and heated at 95ºC for 10 minutes to completely denature the proteins. Around 30µg of the sample was loaded onto an SDS-PAGE setup with Bis-Tris acrylamide gels (4% for stacking and 10% for separating) and run at 120 mV in 1X MOPS buffer (50 mM MOPS, 50 mM Tris-base, 0.1 %SDS, 1 mM EDTA).

After separating the proteins based on molecular weight in the SDS-PAGE, they were transferred to a nitrocellulose membrane (Millipore) using transfer buffer (25 mM Tris, 192 mM Glycine, 20% methanol, 0.1% SDS) for 90 minutes at 90 V. Upon completion of the transfer, membranes were incubated for 20 minutes at room temperature in a blocking solution of 5% non-fat milk in 1X TBS-T (25 mM Tris, 150 mM NaCl, 2 mM KCl and 0.1% Tween20). After blocking, the membrane was treated with primary antibodies diluted in the blocking solution overnight at 4ºC, washed three times with 1X TBS-T, each was involving 5 minutes incubation with shaking, then treated with secondary antibodies coupled with HRP in a similar way for 30 minutes at room temperature. After washing the secondaries in a similar manner, the proteins were detected using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher) on an X-ray film.

Immunoprecipitation

Endogenous pull down

For endogenous ch-TOG IP, U2OS cells were grown in 10cm dishes were collected and lysed using the lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM $MgCl_2$, 0.5% NP40, 1 mM PMSF, and protease inhibitor cocktail) for 20 minutes at 4ºC and the lysates were collected after centrifugation as described above. 50 µL of the lysate was stored for loading as input and the remaining sample was equally divided into two tubes for incubation with the ch-TOG primary antibody (Abcam, ab86073) or unspecific rabbit IgG as control. Following antibody incubation at 4ºC for 30 minutes in a rotating wheel, the

samples were further incubated in a similar manner with Protein G coupled dynabeads (Thermo Fisher Scientific, #10003D). The protein-antibody complexes bound to dynabeads were separated from the solution using a magnet and washed three times with cold lysis buffer. The protein complexes were eluted from the beads by incubating with the 6X sample loading buffer for 20 minutes at room temperature and then analyzed by western blotting.

Anti-FLAG pull-downs

For pull downs involving overexpression, HEK293T cells were used. 48 hrs post transfection, cells were lysed as described above and after storing 50 µL each of the lysates for loading as inputs, the remaining samples were subjected to immunoprecipitation with anti-FLAG antibody conjugated agarose beads (SIGMA, A2220). The bead-antibody-protein complexes were separated using centrifugation at 2000 rpm for 2 minutes at 4ºC, washed three times with lysis buffer and eluted in sample loading buffer as described for analyzing by a western blot.

BioID pull-down

HEK293T cells transiently expressing BirA tagged GCP2/GCP3 constructs were treated with biotin (IBA GmbH, 2-1016-002) at a final concentration of 50 µM for 24 hrs. The cells were then collected and lysed using in SDS lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, and protease inhibitor cocktail) and sonicated. The lysates collected after centrifugation at 16000g for 30 minutes at 4ºC, and 50 µL from each sample were collected as inputs. The remaining lysates were incubated with Streptavidin conjugated sepharose beads (GE Healthcare, GE17-5113-01) for 3 hrs at 4ºC on a rotating wheel, following which the beads-protein complexes were isolated by centrifugation at 2000 rpm for 2 minutes at 4ºC. After three washes with lysis buffer, proteins were eluted from the beads by boiling them in sample loading buffer at 95ºC for 10 minutes and analyzed by western blotting.

Antibodies

The following antibodies were used in the experiments described in this thesis.

Table M2: List of antibodies

Antibody (Clone)	Host species& subtype	Dilution for IF	Dilution for WB	Supplier (Catalogue number)
α-Tubulin (DM1A)	Mouse IgG1	1:2000		Sigma (T6199)
α-Tubulin	Rabbit IgG	1:500		Abcam (ab18251)
α-Tubulin Acetylated	Mouse IgG2b	1:500		Sigma (T6793)
γ-Tubulin (TU-30)	Mouse IgG1	1:500		Exbio (11-465-C100)
γ-Tubulin (GTU-88)	Mouse IgG1		1:10,000	Sigma (T6557)
γ-Tubulin	Rabbit IgG	1:500		Sigma (T5192)
Actin	Mouse IgG1		1:10,000	MP Biomedicals (691001)
CDK5RAP2	Rabbit IgG	1:500		Bethyl (A300-554A)
CEP128	Rabbit IgG	1:500		Bethyl (A303-348A)
ch-TOG	Mouse IgG1	1:100		Santa Cruz Biotechnology (sc-374394)
ch-TOG	Rabbit IgG	1:100	1:2000	Abcam, ab86073
FLAG (M2)	Mouse IgG1		1:10,000	Sigma (A2220)
GAPDH	Mouse IgG1			Santa Cruz Biotechnology (sc-47724)
GCP2	Rabbit IgG		1:2000	In-house
GCP6	Rabbit IgG		1:2000	In-house

GFP	Mouse IgG2a	1:500	1:5000	Life Technologies (A11120)
GFP	Rabbit	1:500	1:5000	Invitrogen (A6455)
GM130	Mouse IgG1	1:500		BD Transduction labs (610822)
GM130-Alexa Fluor 647	Rabbit	1:500		Abcam (ab277924)
NEDD1	Rabbit IgG	1:500	1:2000	In-house
NIN	Mouse IgG2a	1:500		Millipore (MABT29)
NINL	Rabbit IgG	1:200	1:1000	In-house
PCNT	Rabbit IgG	1:500		Novus Bio, (NB100-61071)

The primary antibodies were used in combination with appropriate secondary antibodies conjugated with Alexa Fluor 488/568/647 for immunostaining. All secondaries were purchased from 'Life Technologies' and used at 1:500 dilution. DAPI from Sigma (D9542) was used at 1:50,000 dilution. For western blot, Secondaries conjugated with HRP from 'BD Biosciences' were used at 1:10,000 dilution.

Genome editing

The CRISPR/Cas9 based genome editing was performed by Fabian Zimmermann. To generate CDK5RAP2 KO, single gRNA strategy and for deleting specific regions as in CDK5RAP2ΔCM1, GCP2ΔNTE and GCP2Δexon3, two gRNA to mark the region were used. The gRNAs were administered to RPE1 p53 KO cells using a pX330 based plasmid containing Cas9. Cells expressing the fluorescence reporters (GFP in the case of single gRNA, GFP and mCherry in the case of two gRNAs) were selected by fluorescence assisted cell sorting (FACS Calibur flow cytometer -Becton Dickinson) and were sorted as single cells into 96-well plates to obtain clonal cell lines. The deletion clones were tested by genomic DNA isolation followed by PCR amplification with appropriate primers to identify cells containing homozygous deletions. In cells that produced PCR bands corresponding to homozygous genome editing, the amplified PCR product was cloned into a TOPO vector using TOPO TA cloning kit (ThermoFisher) for sequence verification by Sanger sequencing.

The expression of truncated proteins was further verified by western blotting. CDK5RAP2 KO clones were tested by western blotting to identify clonal lines that lack protein expression.

RESULTS

1

ROLE OF NINL IN MICROTUBULE NUCLEATION

NINL localizes to the subdistal appendages at the centrosome

To study the role of NINL in centrosomal microtubule nucleation, I started by characterizing NINL localization at the centrosome. NINL is known to associate with centrosomes specifically during interphase and gets removed from there at the onset of mitosis by the activity of Plk1 and Nek2 kinases (Casenghi et al., 2003, 2005). This cell cycle dependent centrosomal localization resembles that of known SDA components including the related protein NIN, hence I wanted to check if NINL is also a component of the SDAs.

I used an antibody raised against the endogenous NINL protein to analyze NINL localization in U2OS as well as hTERT-RPE1 cells by immunofluorescence. Super-resolution imaging by 3D-SIM enabled us to obtain sub-centrosomal details, with which I could precisely map the position of NINL along the centriolar wall, marked by staining for acetylated α-tubulin. NINL co-localized with NIN at the SDAs as well as at the centriolar proximal ends (**Figure R1.1**). Notably, the NINL signal at the proximal ends of both centrioles occupy broader area than the NIN signal as seen in the centriolar side views.

NINL is accumulated around duplicating centrioles

Notably, the shape and position of NINL staining along the centrioles were not always as consistent as that of NIN and showed cell to cell variations. In some cells I could also observe labeling that extended outwards, particularly at the proximal centriole ends, and signals that was not in contact with centrioles. This prompted us to speculate that NINL localization at the centrioles may be dynamic. Upon closer examination, I noticed that in most cases such extended signals are associated with centrosomes in early S phase based on the presence of procentrioles revealed by Ac-α-tubulin staining (**Figure R1.2a**). These centrioles also presented stronger NINL accumulations at the proximal ends. Comparison of intensities revealed 1.5-fold increase in NINL levels at the duplicating centrioles compared to centrioles in G1 phase (**Figure R1.2b**).

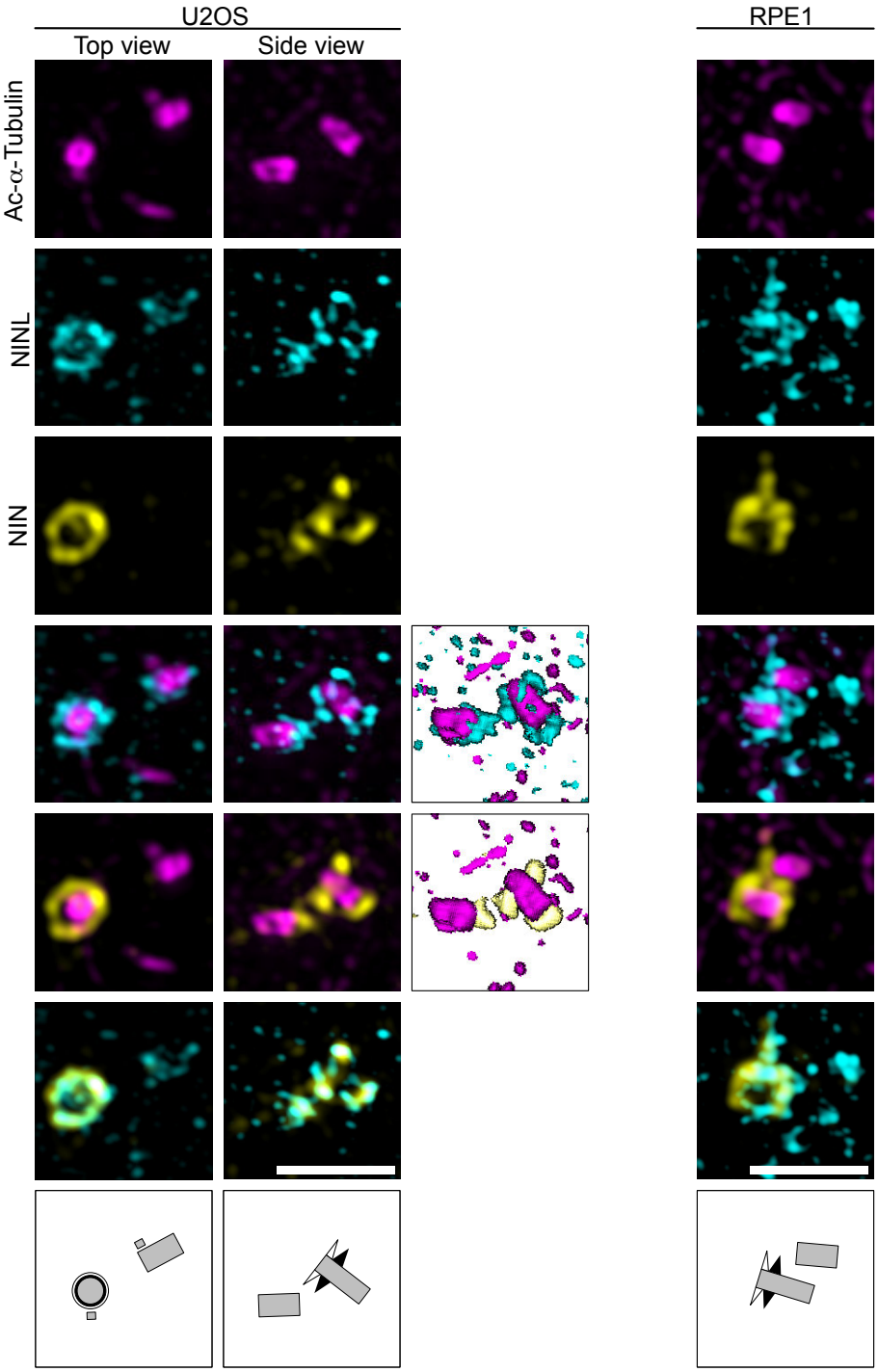


Figure R1.1: NINL localizes to the subdistal appendages

Representative IF images showing the localization of endogenous NINL protein in U2OS and RPE1 cells. Centriole walls are marked by staining for Ac-α-tubulin and NIN is used as a marker for SDAs. Centriole orientations are marked in the cartoon below each panel. 3-dimensional rendering made using ZEISS Zen black software shows the centriolar side view of U2OS cells along with either NINL or NIN as depicted on the right side of the corresponding image. Scale bar 1μm.

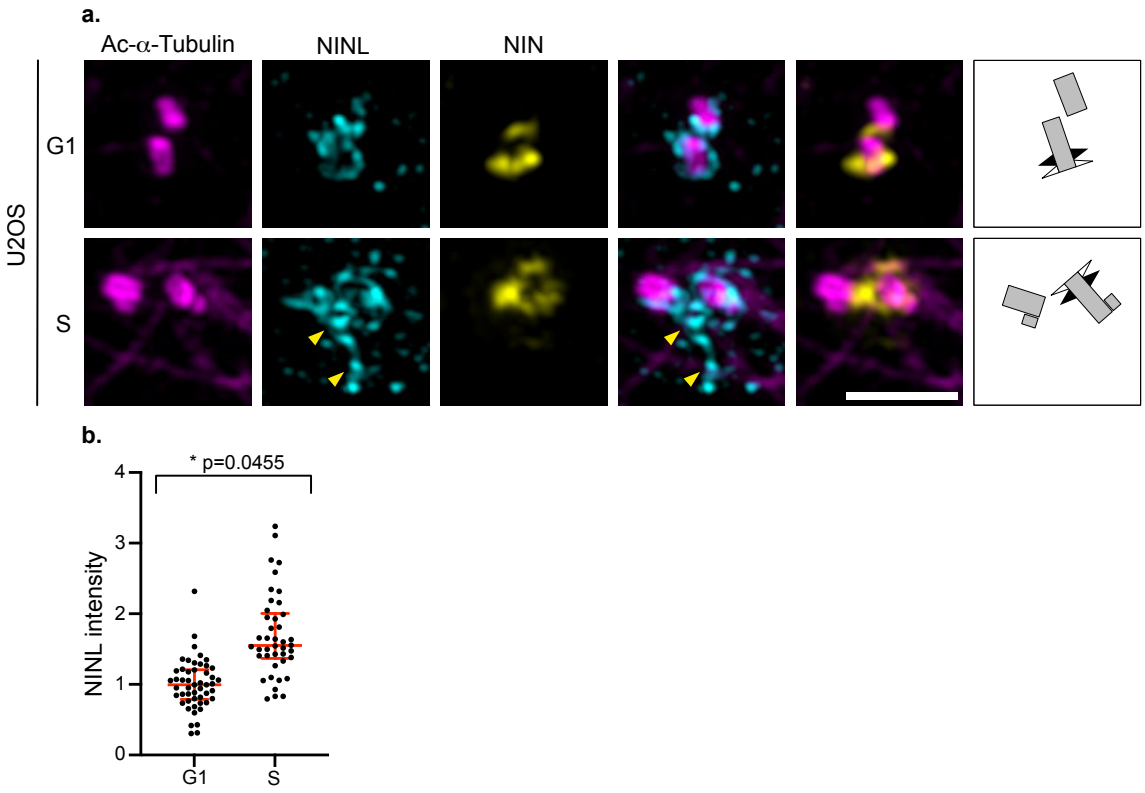


Figure R1.2: NINL accumulates around duplicating centrioles

a. Representative IF images of U2OS cells in G1 or S phase stained for Ac-α-tubulin, NINL and NIN. S phase centrioles show NINL accumulation and long structures at the proximal ends as marked by the yellow arrowheads. Centriole orientations described in the cartoons next to each panel. Scale bar 1μm.

b. Graph showing the intensity of NINL at the centrosome in G1 vs S phase cells as seen in (a), normalized to the average intensity in G1. Three independent experiments, number of centrosomes analyzed: 50 in G1 and 41 in S. Horizontal bars and whiskers indicate median and interquartile range respectively. Statistics performed on the averages from each replicate, p value as shown in the graph.

NINL localizes to the centrosome in the absence of microtubules

NINL's variable distribution at the centrosome and the fact that it is an adapter of the microtubule motor dynein caused us to wonder if the centrosomal distribution of NINL was dependent on microtubules. Upon depolymerization of microtubules with cold treatment, I could not observe any changes in NINL distribution at the centrosome. In particular, the broader proximal localization of NINL at early S phase centrosomes was consistent even in the absence of microtubules (**Figure R1.3**).

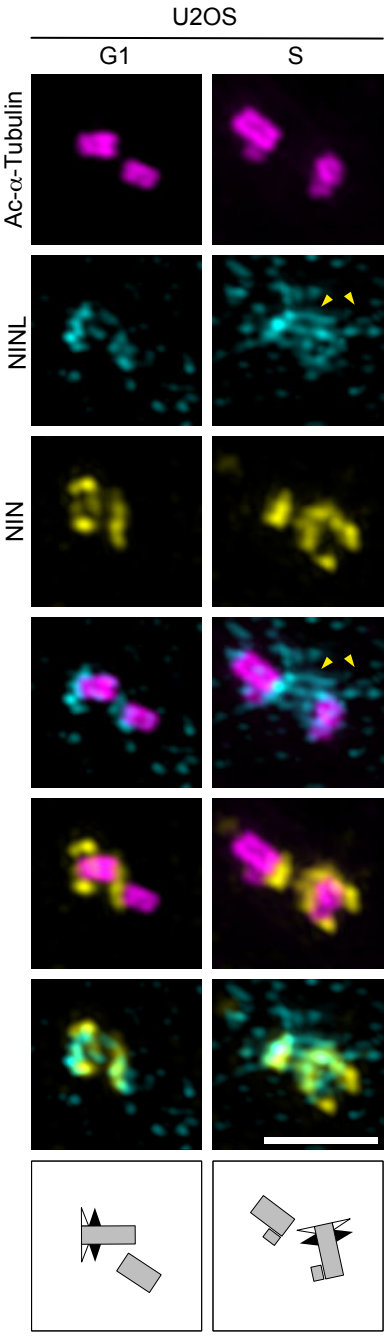


Figure R1.3: NINL localizes to the centrosome in the absence of microtubules

Representative IF images of U2OS cells after microtubule depolymerization with cold treatment demonstrating NINL localization at the centrosomes. Ac-α-Tubulin marks centrioles and NIN used as an SDA marker. Yellow arrowheads mark the extended NINL structures at the proximal ends of S phase centrioles. Centriole orientations are displayed in the cartoons next to each panel. Scale bar 1μm.

Interestingly when I subjected the cells to short microtubule regrowth assays, at the early stages of microtubule growth, NINL appeared to be marking the ends of microtubules at the centrosome (**Figure R1.4a**), although there was no noticeable difference in the overall levels of NINL (**Figure R1.4b**).

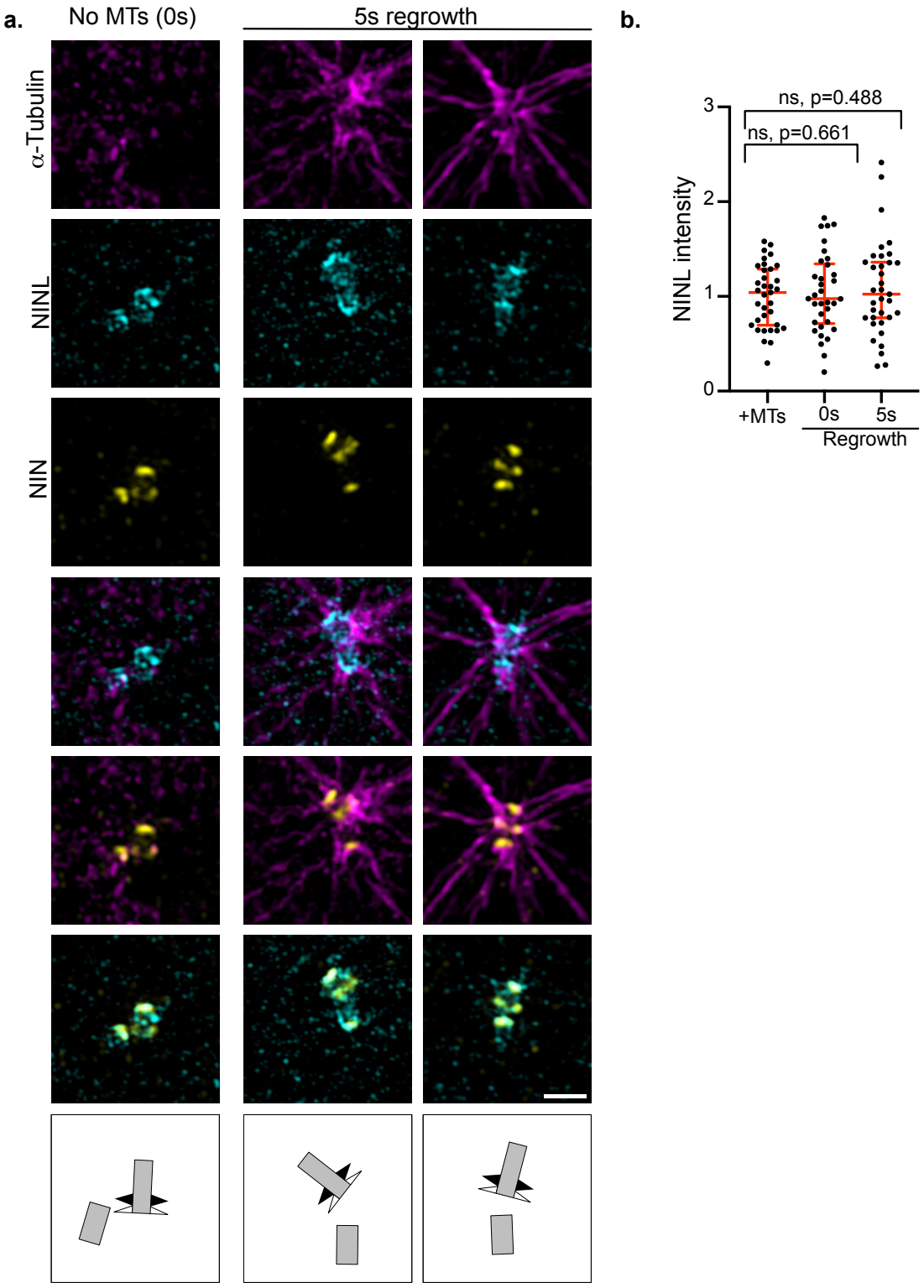


Figure R1.4: NINL associates with the minus ends of newly formed microtubules

a. Representative IF images of U2OS cells subjected to microtubule depolymerization with cold treatment and after 5s of regrowth. α-Tubulin marks microtubules and NIN marks SDAs. Centriole orientations based on NIN staining are shown in the cartoons below each panel. Scale bar 1μm.

b. NINL intensity comparison between different stages of microtubule regrowths. +MT: in the presence of steady state microtubules. 0s, 5s and 10s: time points of regrowth at 37 degrees after microtubule depolymerization by cold treatment. Two independent experiment, 35 centrosomes quantified in each condition. Horizontal bars and whiskers indicate median and interquartile range respectively. Statistics performed on the averages from each replicate, p values as shown in the graph, ns: not significant.

NINL partially colocalizes with dynein at the centrosome

Given NINL is an adapter of the dynein motor, I checked if the NINL localization at the centrosome correspond to that of dynein. By co-staining with antibody against dynein, I observed that NINL partially colocalized with dynein, but also showed localization at additional sites (**Figure R1.5**).

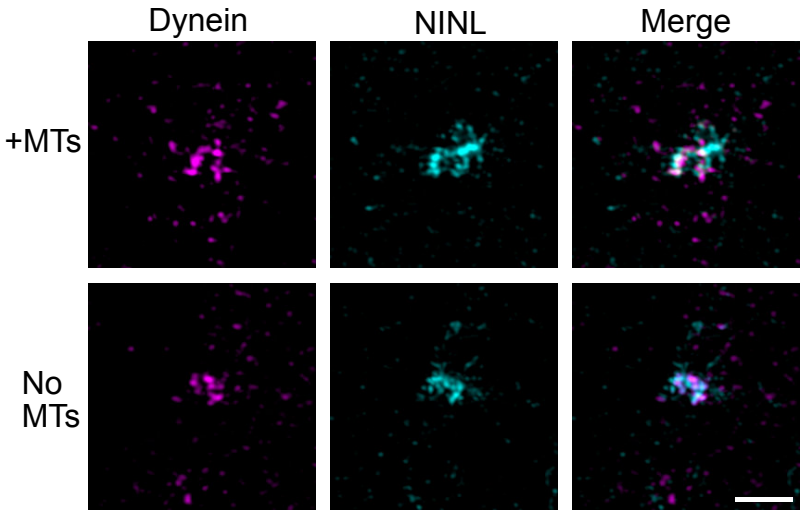


Figure R1.5: NINL partially co-localizes with dynein at the centrosome

U2OS cells co-stained with antibodies against NINL and dynein in the presence of microtubules or after depolymerization by cold treatment. Scale bar 1μm.

So far, based on 3D-SIM imaging, I characterize NINL to be a subdistal appendage protein that accumulates at the proximal ends during early stages of centriole duplication. NINL displays microtubule independent localization at the centrosome, where it also partially colocalizes with dynein, suggesting NINL might have broader centrosomal functions apart from being a dynein adapter.

NINL is not an essential factor for centrosomal microtubule nucleation

To see if NINL is directly involved in microtubule nucleation from the centrosome, we planned to check how this process is affected in the absence of NINL. Unfortunately, none of our siRNAs individually or in combination yielded efficient depletion of the protein at the centrosome. I also tried to generate NINL knock-out cell lines using CRISPR/Cas9 based genome editing but was unsuccessful in obtaining a homozygous knock-out line. Given this difficulty, I decided to check if partial depletion of NINL is sufficient to cause any phenotypes associated with microtubule nucleation. When I tried multiple combinations of siRNAs against NINL, a combination of 3 different siRNAs (referred to as NINL RNAi #2) partially depleted NINL by western blot and resulted in roughly 50% reduction in NINL protein levels at the centrosome (**Figure R1.6**).

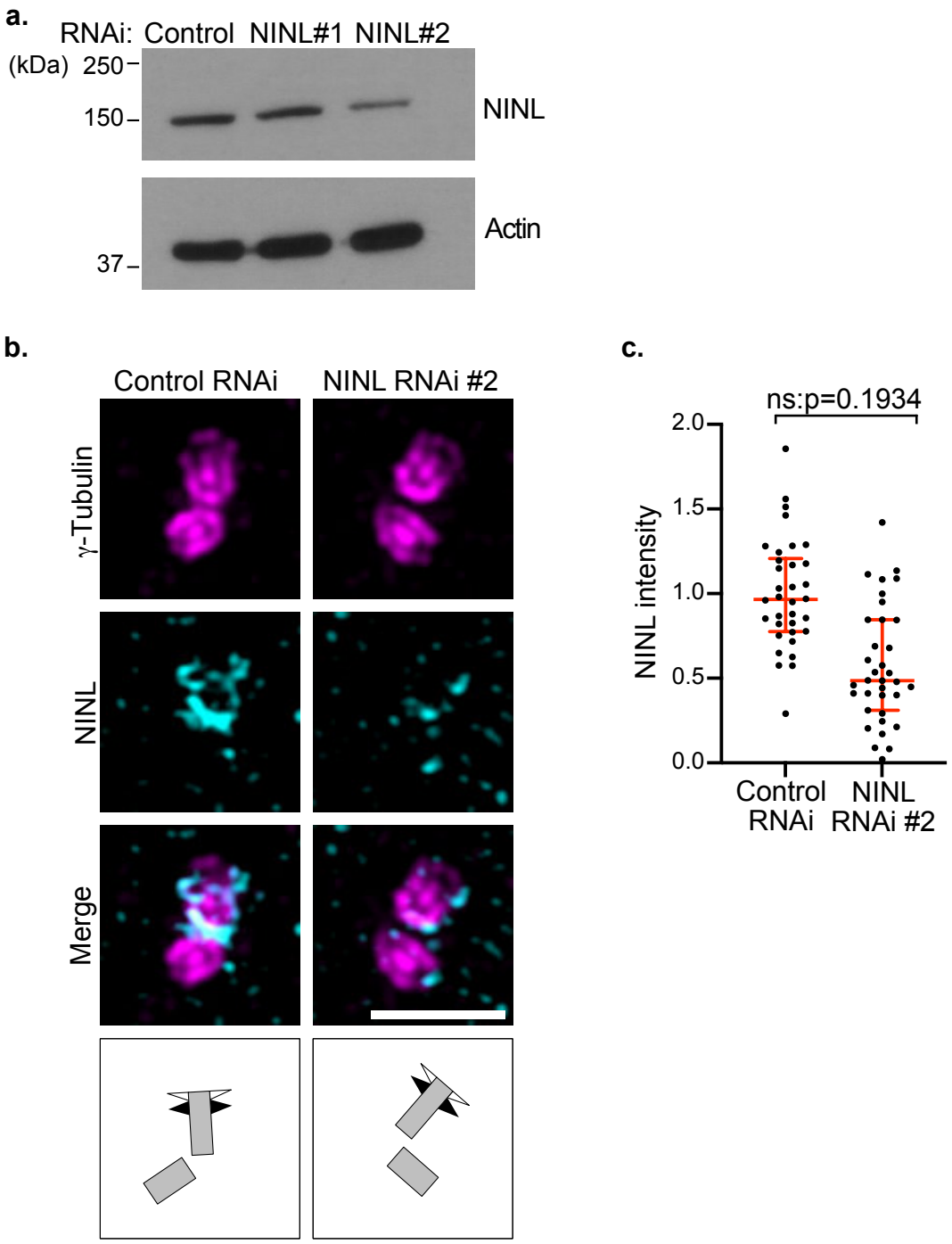


Figure R1.6: NINL is partially depleted by siRNA

a. Western blot of U2OS cell lysates after treatment with control siRNA or two different siRNA combinations against endogenous NINL. Actin serves as a loading control.
b. Representative IF images of U2OS cells after treatment with control siRNA or siRNA combination #2 against NINL. γ-Tubulin marks the centrosome, centriole orientations shown in cartoons next each panel. Scale bar 1μm.

c. NINL intensity comparisons between control siRNA and siRNA combination #2 against NINL in U2OS cells as seen in (**b**). Two independent experiments, 35 centrosomes quantified in each condition. Horizontal bars and whiskers indicate median and interquartile range respectively. Statistics performed on the averages from each replicate, p value as shown in the graph, ns: not significant.

I performed a microtubule regrowth assay in the depleted cells to check for effects in nucleation. The depleted cells showed centrosomal asters at short regrowth time points similar to the control and upon measuring the intensity of the asters I did not observe any reduction. Considering that NINL is not completely removed from the centrosomes, this result does not fully exclude its role in centrosomal nucleation, but as partial depletion of NINL did not have a visible effect on nucleation, NINL may not be a crucial factor in nucleating microtubules at the centrosome (**Figure R1.7**).

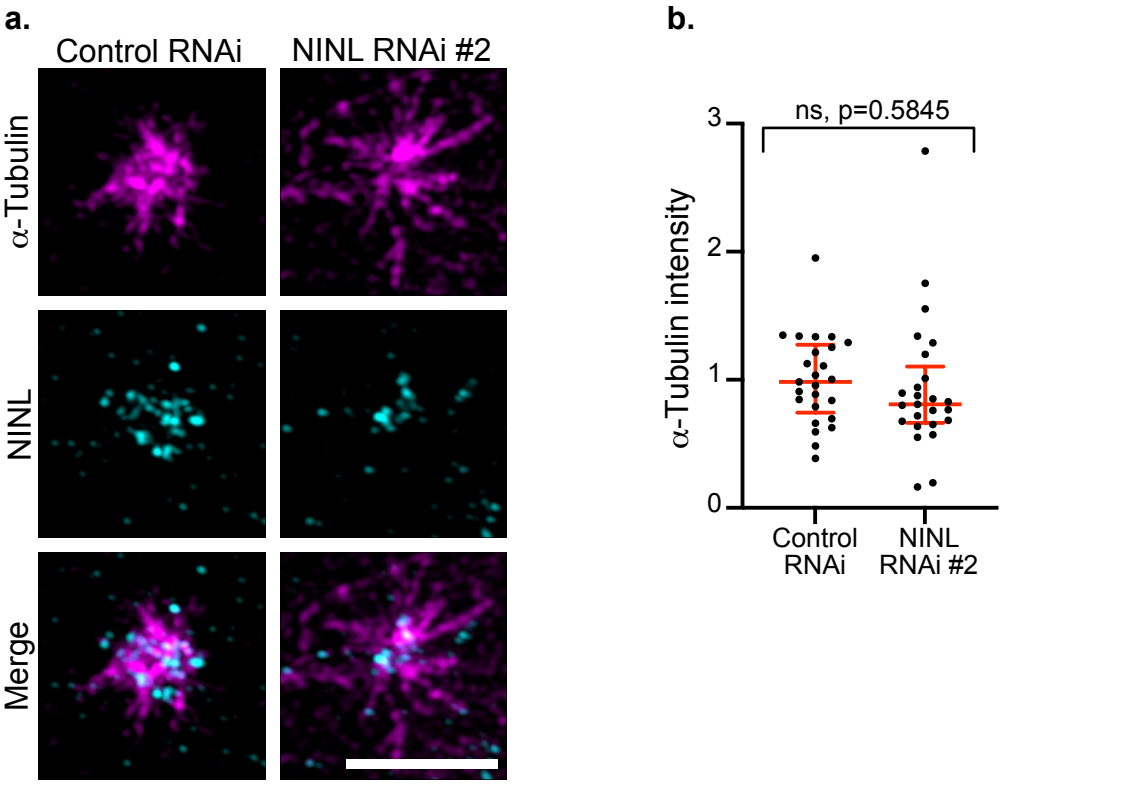


Figure R1.7: NINL partial depletion does not reduce centrosomal microtubule nucleation

a. Representative IF images of U2OS cells subjected to short microtubule regrowth assays after treatment with control siRNA or siRNA combination #2 against NINL. α-Tubulin marks the microtubules. Scale bar 1μm.

b. α-Tubulin intensity comparisons between control siRNA and siRNA combination #2 against NINL in U2OS cells as seen in (a). Two independent experiments, 25 centrosomes quantified in each condition. Horizontal bars and whiskers indicate median and interquartile range respectively. Statistics performed on the averages from each replicate, p value as shown in the graph, ns: not significant.

Ectopic MTOC generation by NINL involves ch-TOG recruitment

Our interest in testing the role of NINL in microtubule nucleation derives from the observed ability of NINL N-terminus to form ectopic MTOCs in the cytoplasm upon overexpression (Casenghi et al., 2003). In the lab, work from a previous student identified minimal regions within the N-terminus of NINL protein that contribute to generating ectopic MTOCs. When targeted to the mitochondrial surface, the region containing amino acids 1-287 was able to recruit γTuRC but unable to nucleate microtubules in a regrowth assay. On the other hand, a larger construct with amino acids 1-442 was sufficient to recruit γTuRC as well as activate nucleation at this site (as described in the introduction **Figure I2.2**). We wondered if activation of nucleation from γTuRC at the mitochondrial surface requires additional factors that are recruited only by the larger 1-442 construct.

Among several NINL interactors identified in a BioID experiment, we were interested in the microtubule polymerase ch-TOG as it is the human orthologue of *Xenopus* XMAP215, recently shown to promote nucleation from γTuRC *in vitro* (Thawani et al., 2018, 2020; Thawani & Petry, 2021). In addition, the microtubule associated protein MAP7D3 was also an interesting candidate. MAP7D3 has been reported to be a microtubule stabilizing factor with possible roles in nucleation, but its functions have not been studied well (Sun et al., 2011; Yadav et al., 2014).

We wondered if these two proximal interactors might play a role in the formation and/or function of the MTOCs formed by targeting NINL to ectopic sites. To test that, I checked if these proteins were recruited to the mitochondrial surface by the largest NINL fragment and if the differential ability of smaller NINL constructs in nucleating microtubules at the ectopic site may be linked to their ability to recruit these factors. For this comparison, I expressed the mitochondrial targeting versions of NINL N-terminus (amino acids 1-702), NINL 1-442 and NINL 1-287 and checked for the recruitment of ch-TOG and MAP7D3.

Interestingly, MAP7D3 was strongly associated with all three NINL fragments at the mitochondria, suggesting that it interacted with the most N-terminal 1-287 region of NINL (**Figure R1.8**). However, since this fragment did not induce nucleation, MAP7D3 binding appears not to be sufficient for stimulating nucleation.

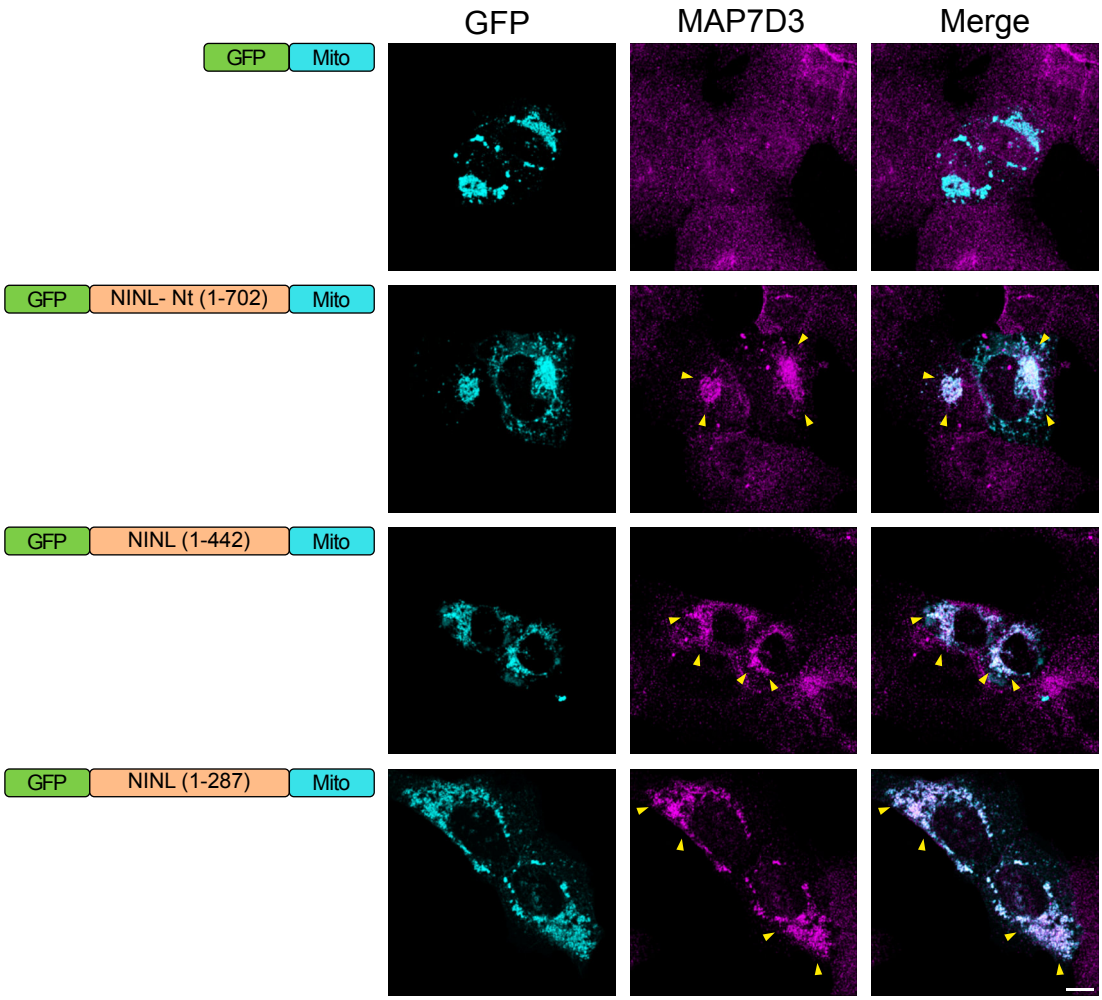


Figure R1.8: NINL 1-287 is sufficient to recruit MAP7D3 to the mitochondria

Representative IF images of MAP7D3 recruitment to mitochondrial surface by NINL minimal constructs. U2OS cells transiently transfected with mitochondrial targeting versions of NINL N-terminus (1-702), NINL 1-442 and NINL 1-287 were imaged by staining with antibodies against the GFP tag and endogenous MAP7D3 protein. Yellow arrowheads mark the area of colocalization. Scale bar 10μm.

Staining for ch-TOG in these conditions produced a strong non-specific signal from the nucleus, making it difficult to observe its recruitment to the mitochondria. While it was observed at low amounts at the mitochondrial surface upon targeting the larger 1-702 and 1-442 fragments, it was completely absent when 1-287 fragment was targeted to the ectopic site (**Figure R1.9**). Together with the observation from previous work in the lab that microtubule nucleation at the mitochondrial surface upon targeting NINL N-terminus (1-702) is strongly reduced in ch-TOG depleted cells (Paz Domínguez, 2021), it can be inferred that ch-TOG is an essential component for the nucleation activity of this ectopic MTOC.

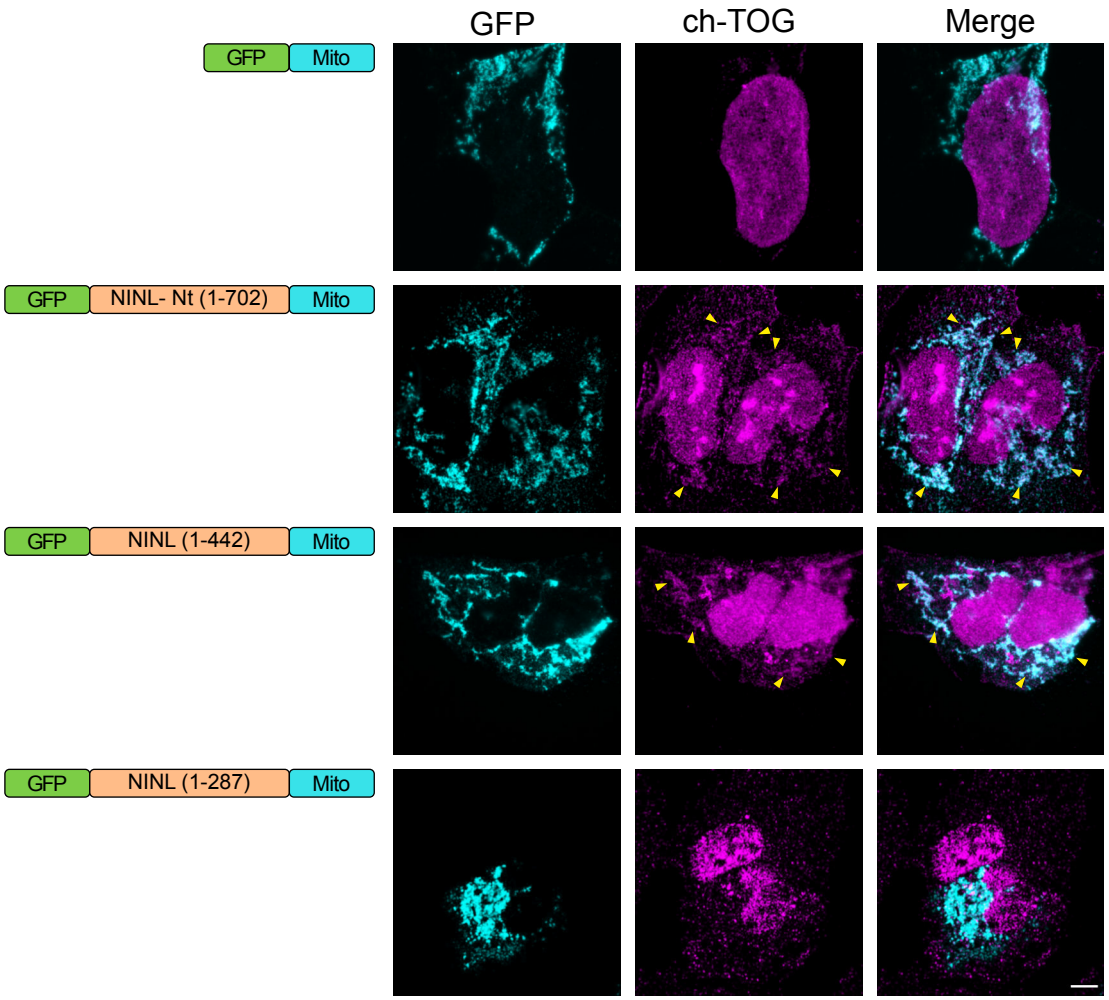


Figure R1.9: NINL 1-287 does not recruit ch-TOG to the mitochondria

Representative IF images of ch-TOG recruitment to mitochondrial surface by NINL minimal constructs. U2OS cells transiently transfected with mitochondrial targeting versions of NINL N-terminus (1-702), NINL 1-442 and NINL 1-287 were imaged by staining with antibodies against the

GFP tag and endogenous ch-TOG. Yellow arrowheads mark the area of colocalization. Scale bar 10μm.

In summary, the N-terminal 1-442 region of NINL is sufficient to assemble MTOCs at ectopic sites but is not sufficient for nucleation. This activity depends on ch-TOG, which is recruited by this region in NINL.

2

ch-TOG IN MICROTUBULE NUCLEATION AT CENTROSOMAL AND NON-CENTROSOMAL MTOCS

ch-TOG at the centrosome: localization, γTuRC recruitment and microtubule nucleation

Studies on microtubule polymerases of the XMAP215 family in yeast and in *Xenopus* egg extract *in vitro* suggest a role in microtubule nucleation in cooperation with γ-tubulin complexes (Popov et al., 2002; Flor-Parra et al., 2018; Gunzelmann et al., 2018; Thawani et al., 2018; Consolati et al., 2020). We were interested to see if ch-TOG, the human orthologue of XMAP215 was involved in microtubule nucleation in human cells. Work from a post-doctoral fellow in the lab, Aamir Ali, identified some of the functional contributions of ch-TOG at the centrosome.

One surprising observation was the way ch-TOG localizes to the centrosome. Firstly, we were surprised that being a potential nucleating factor, ch-TOG at the centrosome did not localize to the PCM, where a majority of the microtubules are nucleated, but was present at the SDAs. Interestingly, upon depolymerization of microtubules by either cold or nocodazole treatment, ch-TOG accumulated at the PCM. When these cells were subjected to microtubule regrowth assay and imaged at specific time points, the ch-TOG at the PCM appeared to be dispersing as the microtubules grew outwards until finally only the SDA-associated ch-TOG remained (**Figure R2.1**).

Co-staining with α-tubulin revealed that this dispersal of ch-TOG during microtubule regrowth occurs by its association with the plus ends of newly formed microtubules (**Figure R2.1**_lower panel). This demonstrates the dynamic localization of ch-TOG at the centrosome, whereby ch-TOG accumulates at the PCM in the absence of microtubules and at the early stages of microtubule nucleation and then moves away along the growing microtubule ends. The SDA associated pool of ch-TOG appears to be stably present.

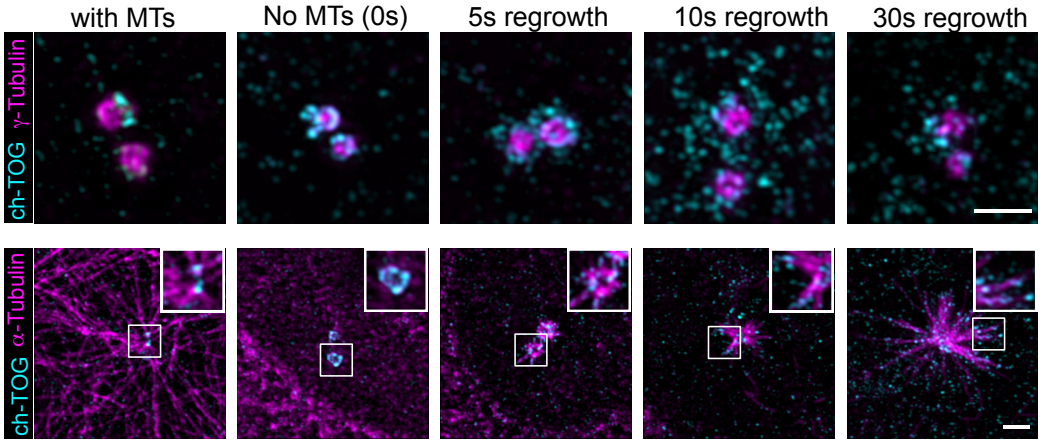


Figure R2.1: ch-TOG shows microtubule dependent dynamic localization at the centrosome

U2OS cells stained for endogenous ch-TOG along with either γ-tubulin (upper panel) or α-tubulin (lower panel) in the presence of microtubules (with MTs), after depolymerization (No MTs) and at different stages of microtubule regrowth. Scale bar 1μm.

Interestingly, siRNA mediated knock-down of ch-TOG resulted in the loss of γTuRC from the PCM in interphase centrosomes in U2OS cells, but did not affect γ-tubulin inside the centriolar lumen (**Figure R2.2a**). In addition, ch-TOG depletion heavily reduced microtubule nucleation at the centrosome (**Figure R2.2b**). Apparently, this transient association of ch-TOG to the PCM is essential in the stable integration of γTuRC and microtubule nucleation at this site.

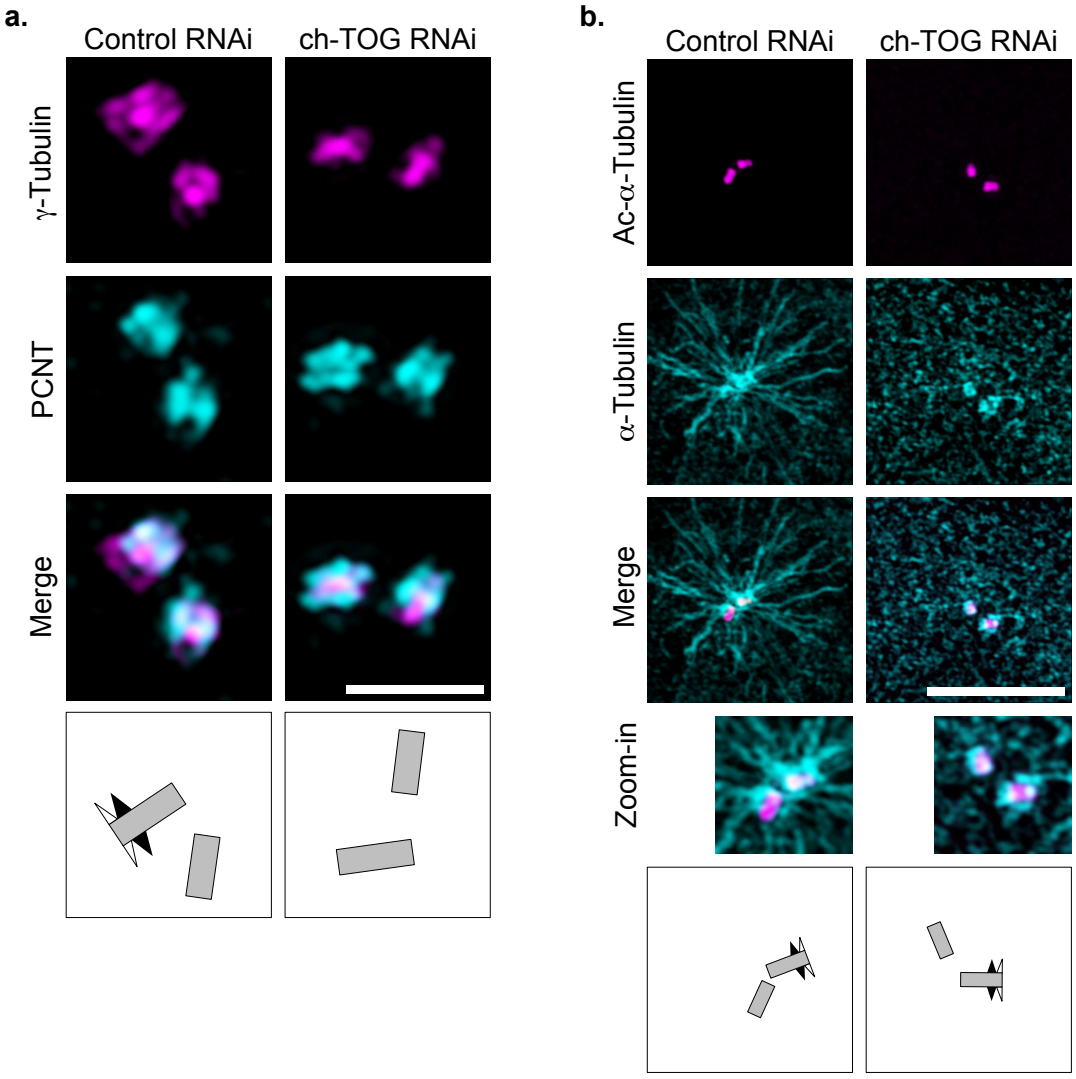


Figure R2.2: ch-TOG depletion impairs γ-tubulin PCM localization and microtubule nucleation at the centrosome

U2OS cells treated with control siRNA or siRNA against ch-TOG.
a. staining of γ-tubulin and PCM marker pericentrin (PCNT) showing the loss of γ-tubulin from the PCM upon ch-TOG depletion.
b. microtubule regrowth assay, 5s regrowth after cold depolymerization. α-Tubulin marks the microtubules and Ac-α-tubulin marks the centrioles. Scale bar 1μm.

ch-TOG has a stronger effect on γTuRC PCM localization in interphase compared to CDK5RAP2

As this is the first report of the role of ch-TOG in centrosomal γTuRC recruitment, we were curious as to how the contribution from ch-TOG compares with the known γTuRC recruiting protein CDK5RAP2.

To address this, I used hTERT-RPE1 cells that are CRISPR edited to knock-out the CDK5RAP2 gene, provided by the group of Anna Akhmanova (University of Utrecht, Netherlands). First, I verified the effect of ch-TOG depletion in γTuRC recruitment in wild type RPE1 cells. In these cells, as in the U2OS, γTuRC from the PCM region was lost upon ch-TOG depletion while the luminal signal remained, giving rise to ~50% reduction in overall γ-tubulin signal. The CDK5RAP2 KO cells showed slightly reduced levels of γ-tubulin at the centrosome, and upon ch-TOG depletion the levels further reduced predominantly in the PCM (**Figure R2.3**). Thereby our results demonstrate that in interphase centrosomes, ch-TOG is more important than CDK5RAP2 in the stable localization of γTuRC to the PCM.

ch-TOG is dispensable for γTuRC recruitment to mitotic centrosomes

As we have observed the effect of ch-TOG in γTuRC recruitment to the PCM during interphase, we wondered whether this dependency is also a true during mitosis, a stage characterized by expanded PCM, increased γTuRC accumulation and enhanced microtubule nucleation at the centrosome. Interestingly, we did not observe any significant reduction in γ-tubulin levels at the mitotic poles upon ch-TOG depletion (**Figure R2.4**). While this would suggest the presence of ch-TOG independent pathways of γ-TuRC recruitment to mitotic centrosomes, with PCM proteins such as CDK5RAP2 taking over the major roles, it does not fully exclude the contributions from ch-TOG. Instead, during mitosis, there could be multiple redundant factors at play to ensure a consistent supply of microtubules necessary to carry out the process of cell division.

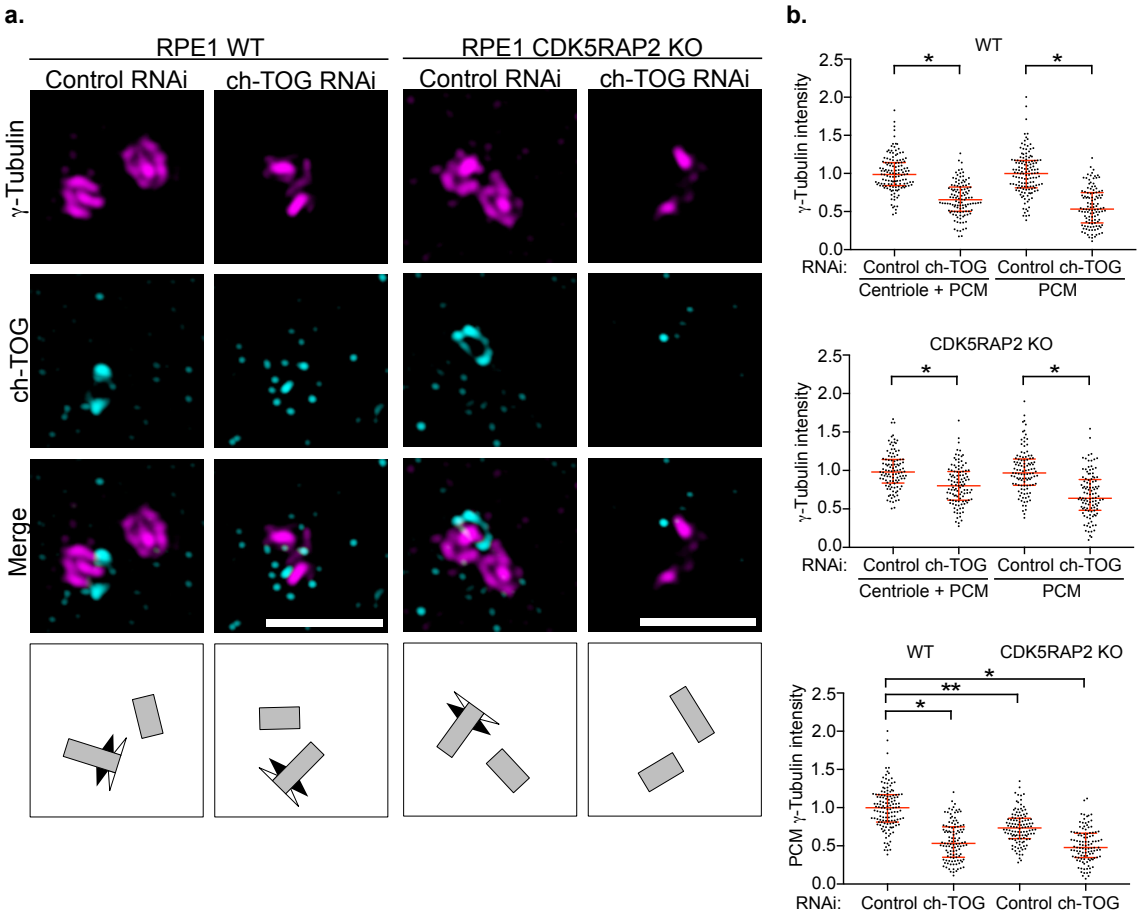


Figure R2.3: ch-TOG has a stronger effect on γTuRC PCM localization in interphase compared to CDK5RAP2

a. Representative IF images of RPE1 WT or CDK5RAP2 KO cells treated with control or ch-TOG siRNA stained with antibodies against γ-tubulin and ch-TOG. Scale bar, 1μm.

b. Intensities of centrosomal γ-tubulin staining in cells as in (**a**), quantified for the entire centrosome region or specifically for the PCM, were normalized to the mean of the respective control and plotted. N=3 experiments, total number of cells analyzed:

RPE1 WT: 71 (control RNAi), 57 (ch-TOG RNAi), *p=0.0497 (γ-tubulin at centriole + PCM), *p=0.0276 (γ-tubulin at PCM)

RPE1 CDK5RAP2 KO: 69 (control RNAi), 59 (ch-TOG RNAi), *p=0.0422 (γ-tubulin at centriole + PCM), *p=0201 (γ-tubulin at PCM)

*p=0.0319 (γ-tubulin at PCM in RPE1 WT vs CDK5RAP2 KO control cells), *p=0.0135 (γ-tubulin at PCM in RPE1 WT control vs CDK5RAP2 KO ch-TOG depleted cells).

The horizontal bars and whiskers indicate median and interquartile range, respectively.

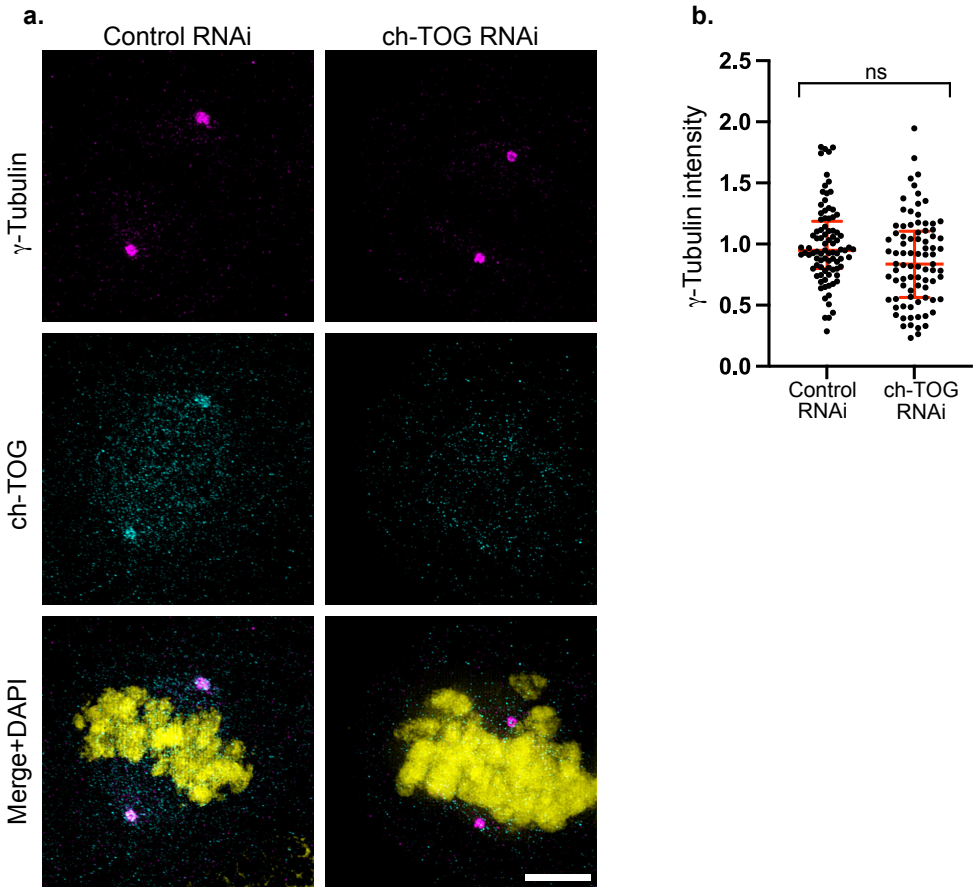


Figure R2.4: ch-TOG is dispensable for γTuRC PCM recruitment to mitotic centrosomes

a. Representative IF images of mitotic U2OS cells treated with control or ch-TOG siRNA stained with γ-tubulin and ch-TOG antibodies. DAPI was used to label DNA.
b. Intensities of centrosomal γ-tubulin staining in cells as in (**a**) were normalized to the mean of the control and plotted. N=3 experiments, total number of centrosomes analyzed: 44 (control RNAi) and 42 (ch-TOG RNAi); p=0.0893 (ns: not significant). The horizontal bars and whiskers indicate median and interquartile range, respectively. Scale bar 5μm.

ch-TOG does not form a stable complex with γTuRC

As we have established that γTuRC PCM localization during interphase is dependent on ch-TOG, we wondered if this involved a direct interaction between the two, whereby ch-TOG binds to the γTuRC and recruit it to the centrosome. To answer this, I performed coimmunoprecipitation assay where I used antibody against endogenous ch-TOG for pull down and analyzed by western blot to check the presence of γTuRC components. Interestingly, neither γ-tubulin, GCP2 or NEDD1 were present in the immunoprecipitates

(**Figure R2.5a**). This suggests that ch-TOG does not form a stable complex at least not with the cytoplasmic pool of γTuRC.

In previous work from my group ch-TOG was identified as one of the proximal interactors of γTuRC components in a mass-spectrometry analysis of BioID samples. In contrast to immunoprecipitation, BioID is suited to detect also weak or transient interactions. I tested if the interaction between γTuRC and ch-TOG by BioID can also be visualized in a western blot. I performed a small scale BioID by expressing BirA fused versions of GCP2 or GCP3 in HEK293T cells and after 24h incubation with biotin, collected the lysates and subjected these to a pull-down using streptavidin-coupled sepharose beads. When the eluate was analyzed by western blot, we could clearly detect ch-TOG in the sample, suggesting ch-TOG is a proximal interactor of γTuRC (**Figure R2.5b**).

To verify that the overexpression of GCPs did not contribute to this observed interaction, I used the same GCP3 construct used for the BioID and performed a co-IP using the FLAG tag present in them but was unable to detect ch-TOG in the sample (**Figure R2.5c**). This suggest that ch-TOG does not form a stable complex with γTuRC but interacts only weakly or transiently with γTuRCs.

Taken together with the observation that ch-TOG localizes to the PCM only transiently, this would implicate a very dynamic interaction of ch-TOG with γTuRC, possibly during the very early stages of microtubule nucleation, before it disperses from the PCM along with the outgrowing microtubule plus ends.

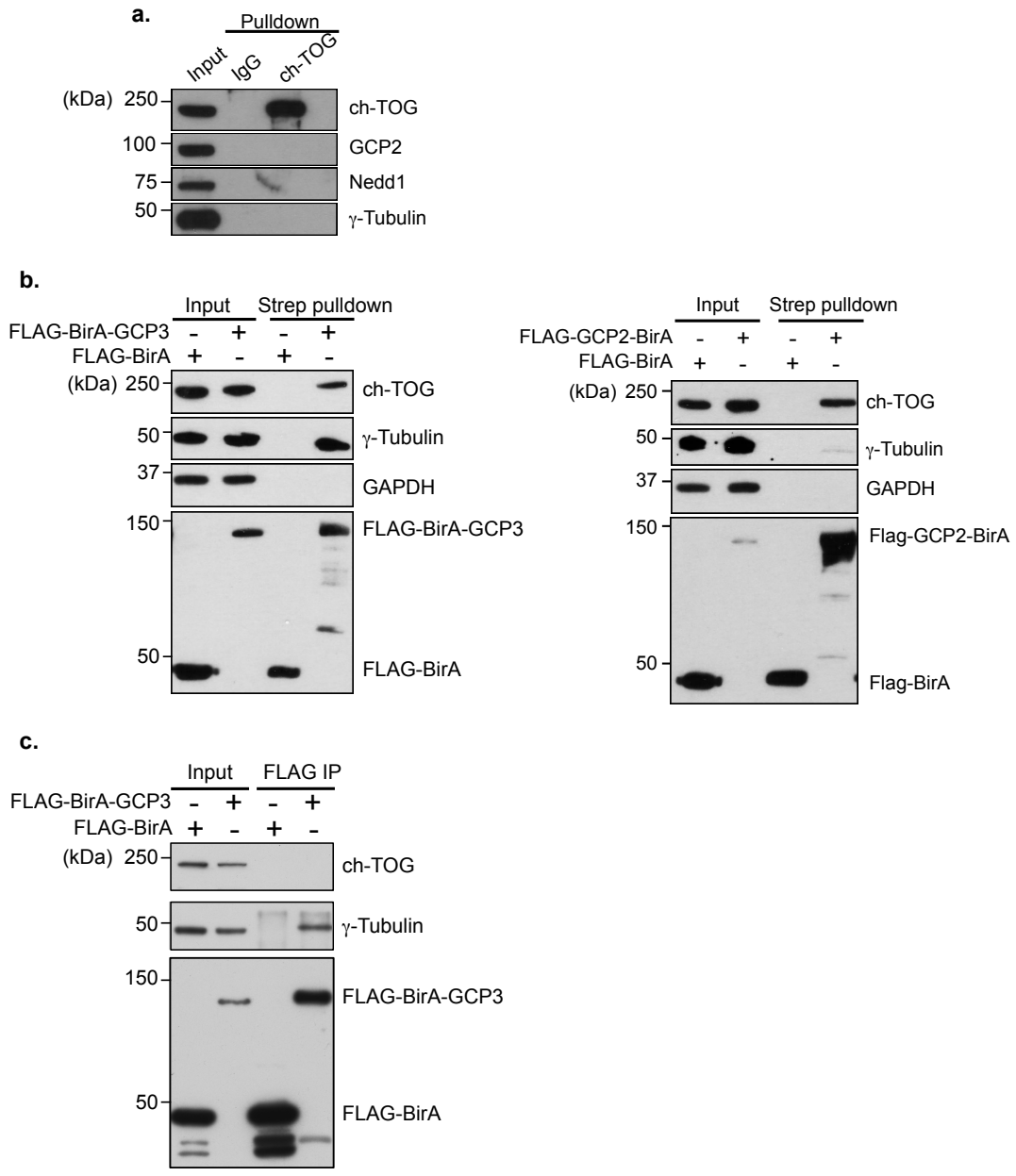


Figure R2.5: ch-TOG does not form a stable complex with γTuRC

a. U2OS cell lysates subjected to immunoprecipitation using antibody against ch-TOG or unspecific IgG as control were analyzed in a western blot by probing for the indicated proteins

b. BioID experiment wherein HEK293T cells transfected with FLAG-BirA-GCP3 or FLAG-GCP2-BirA constructs were incubated with biotin for 24h and subjected to pulldown using streptavidin coated sepharose beads, and analyzed in a western blot by probing for the indicated proteins. FLAG-BirA transfected cells were used as negative control.

c. HEK293T cells transfected with FLAG-BirA or FLAG-BirA-GCP3 as in (**b**) were subjected to immunoprecipitation using Anti-FLAG antibody coated beads and analyzed in a western blot by probing for the indicated proteins.

ch-TOG localizes to the PCM independent of its SDA localization

Given that the ch-TOG association with the SDAs is quite stable unlike the microtubule dependent transient localization at the PCM, we asked whether the SDA pool of ch-TOG is directly involved in the PCM localization as well. To test this, we decided to perturb the SDAs themselves by the knock-down of the inner structural component CEP128 and check if this affects ch-TOG recruitment to the PCM. In the control cells, as established before, ch-TOG was present at the SDAs in the steady state and upon microtubule depolymerization, accumulated towards the proximal ends of both centrioles. Upon CEP128 depletion, the SDA structure was completely lost as seen by NIN staining, wherein NIN is associated only with the proximal centriole ends. The SDA associated ch-TOG was also lost in these cells. Strikingly, when subjected to microtubule depolymerization, ch-TOG appeared accumulate at the centrosome, while NIN remained the same (**Figure R2.6**). Notably, the newly recruited ch-TOG was not limited to the areas of NIN localization. This result has been observed in two independent experiments, but further analysis and quantifications are needed to confirm the accumulation ch-TOG and carefully evaluate the sub-centrosomal locations the protein gets recruited to.

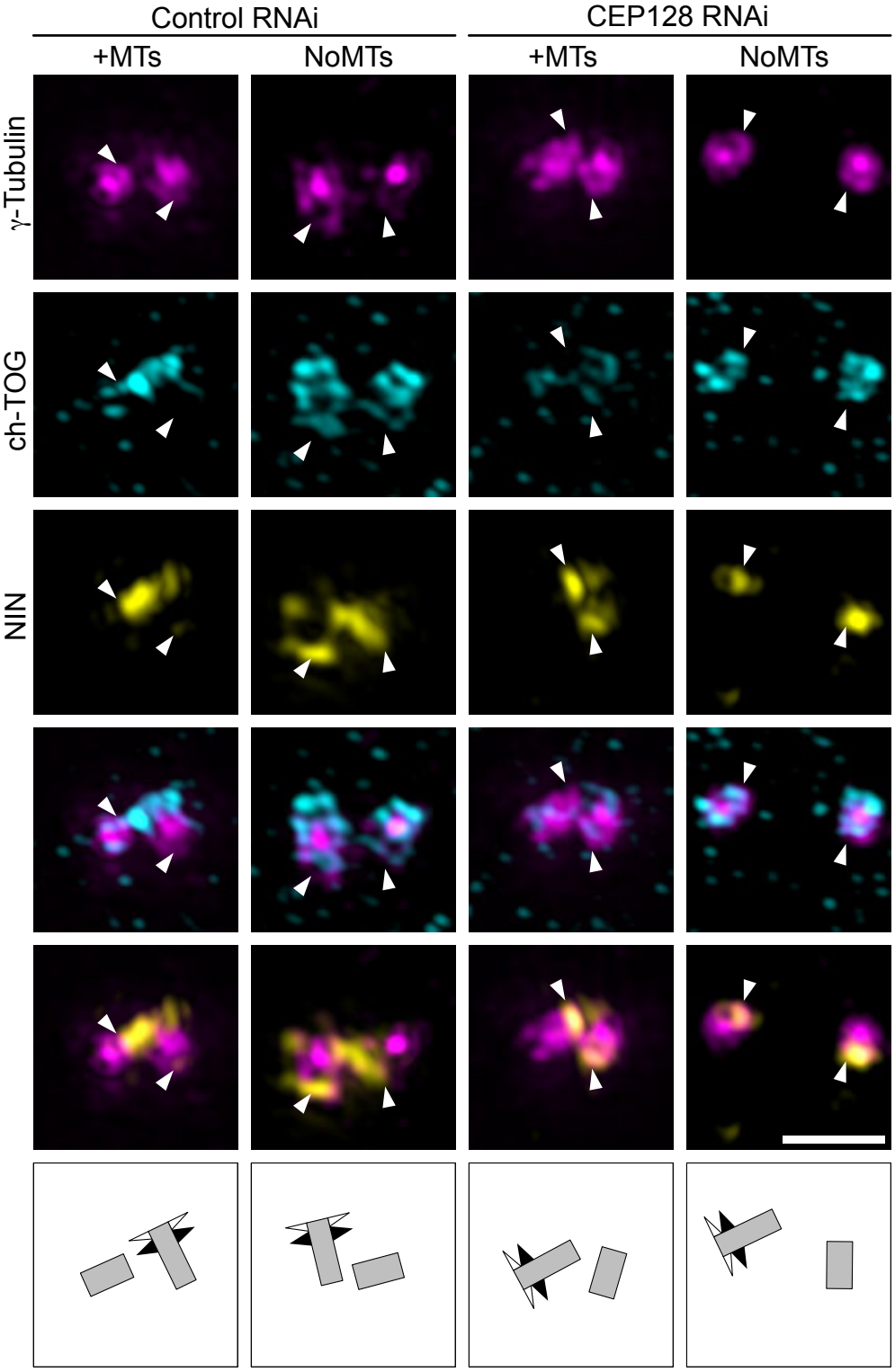


Figure R2.6: ch-TOG PCM localization is independent of its SDA localization

Representative IF images U2OS cells treated with control or CEP128 siRNA in the presence of microtubules (+MTs) and after cold depolymerization (NoMTs). White arrowheads mark the PCM region of both the centrioles. Scale bar 1μm.

ch-TOG promotes microtubule nucleation at the Golgi

While the centrosome is the prominent MTOC in cycling cells, most cell types also harbor non-centrosomal sites of microtubule nucleation and anchoring. RPE1 cells rely highly on Golgi derived microtubules. We wondered whether ch-TOG is also involved in microtubule nucleation at these non-centrosomal MTOCs. I used triple labelling of ch-TOG, α-tubulin and the cis-Golgi marker GM130 in cells subjected to short microtubule regrowth to see if ch-TOG is associated with these nucleation sites. Given the transient association of ch-TOG at the centrosomal nucleation sites, we decided to use nocodazole based depolymerization of microtubules, that will allow a slower rate of nucleation and growth in a regrowth assay. This treatment also fragmented the Golgi, making it easier to visualize individual nucleation sites.

Following drug washout and regrowth at 37°C, earliest stages of microtubule assembly at the Golgi stacks were observed at 5s, where they were too small to have the elongated microtubule structure, but was distinguishable based on their brighter signal compared to the background. ch-TOG also formed bright puncta at the ends of this early microtubules, both of which colocalized with the Golgi marker. At 10s of regrowth, short microtubules were more clearly visualized. While ch-TOG was observed associated with a few of the short microtubules formed at the Golgi, it was not always distinguishable from the cytoplasmic signal (**Figure R2.7**).

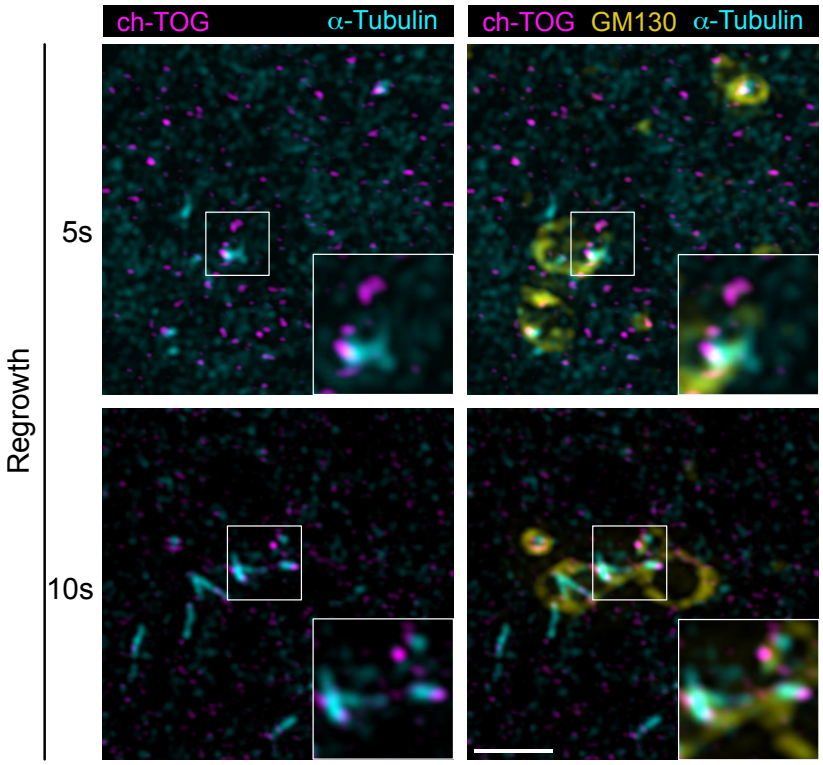


Figure R2.7: ch-TOG associates with Golgi derived microtubules

Representative IF images of early stages of microtubule regrowth from the Golgi in RPE1 cells. After treating with nocodazole to depolymerize the microtubules and drug washout, cells were subjected to short regrowth assay for the indicated time periods. ch-TOG puncta brighter than the background is associated with some of the short microtubules. Scale bar, 2μm.

Upon ch-TOG depletion in RPE1 cells, the number of microtubules generated at the Golgi was also highly reduced, suggesting a role of ch-TOG in microtubule nucleation at this non-centrosomal MTOC (**Figure R2.8**). The effect was quantified by counting the number of microtubules surrounding the Golgi area in the control and depleted cells, after a 5s regrowth.

In summary these results identify ch-TOG as a crucial factor in microtubule nucleation at the interphase centrosomes and Golgi, through transient association with the γTuRC, a process that also promotes stable integration of γTuRC at the interphase PCM.

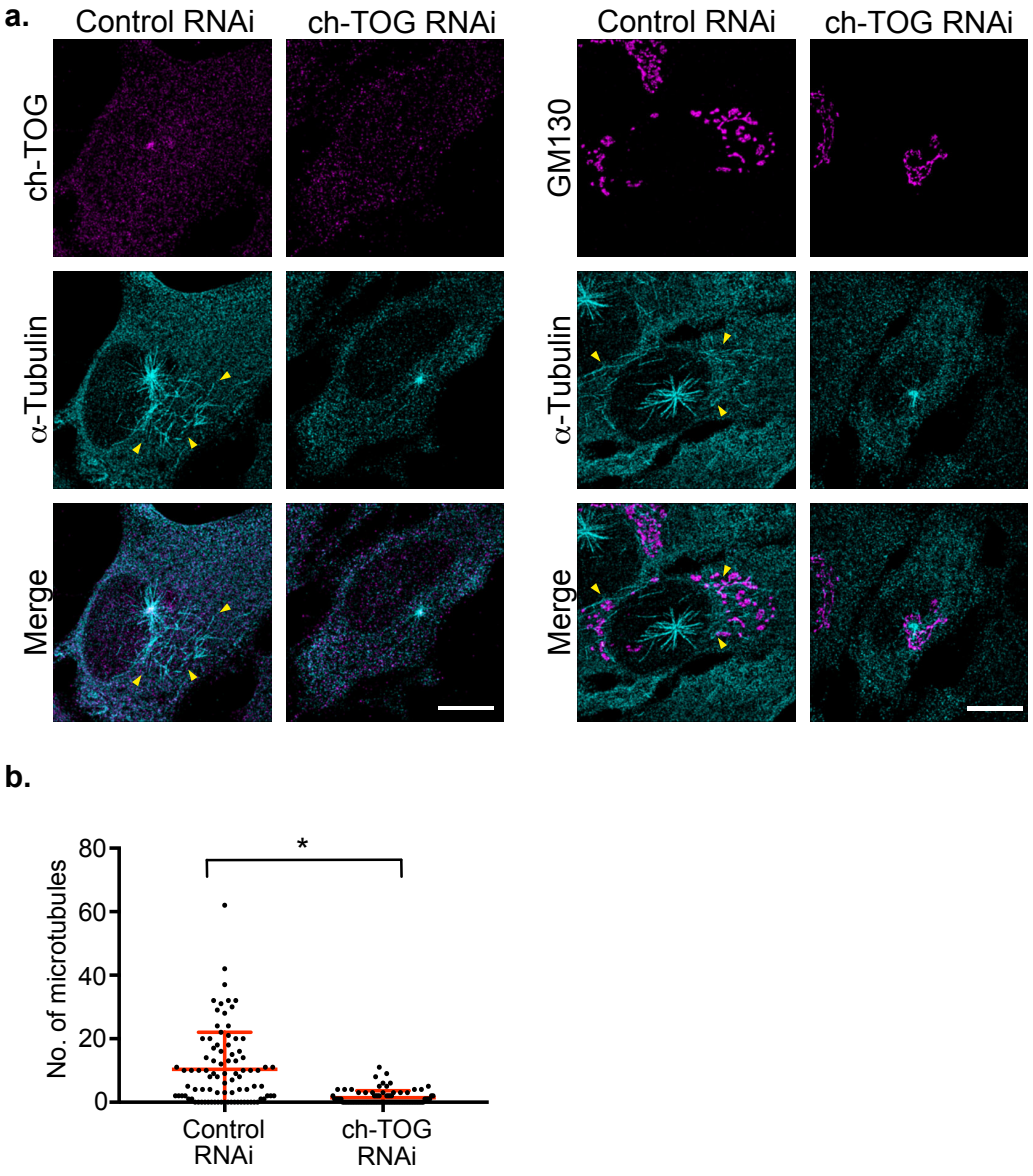


Figure R2.8: ch-TOG promotes microtubule nucleation from Golgi

a. RPE1 cells treated with control or ch-TOG siRNA were subjected to microtubule regrowth following depolymerization by nocodazole treatment. Microtubules were visualized by staining for α-Tubulin along with either ch-TOG or the cis-Golgi marker GM130 to identify Golgi derived microtubules in ch-TOG depleted cells. Yellow arrowheads mark the Golgi-derived microtubules Scale bar 10μm.

b. Number of microtubules in the Golgi area marked by GM130 staining was counted in control and ch-TOG siRNA treated cells and plotted. N = 3 experiments, total number of cells analyzed: 90 in each condition, *p = 0.0207. The horizontal bars and whiskers indicate median and interquartile range, respectively.

3

γTuRC-CM1 INTERACTION IN FACILITATING MICROTUBULE NUCLEATION

Loss of CM1 motif does not disrupt γTuRC localization or microtubule nucleation at the centrosome

The binding of CDK5RAP2 via its CM1 domain has been proposed to activate the γTuRC. The presence of just the CM1 region, a sequence of ~50 amino acids, was shown to enhance nucleation *in vitro* as well as in the cytoplasm upon its overexpression (Choi et al., 2010). To investigate if and how CM1 binding to γTuRC influences microtubule nucleation at the interphase centrosome, I decided to check if the lack of this domain has any effect on centrosomal nucleation activity.

I used a cell line generated by a lab member Fabian Zimmermann that was edited by CRISPR to have the endogenous sequences corresponding to the CM1 region deleted, hereafter referred to as the CDK5RAP2ΔCM1. To avoid any p53 mediated cell cycle arrest or apoptosis resulting from the manipulation of centrosomal proteins, we specifically generated these cell lines in a p53 null (p53 KO) background. In addition, a CDK5RAP2 knock out cell line (referred to as CDK5RAP2 KO) in the p53 null background was also used for comparison.

Firstly, I looked at the centrosome in these cells by staining for γ-tubulin. During interphase, both CDK5RAP2 KO and CDK5RAP2ΔCM1 cell lines appeared to have γ-tubulin localization at the centrosome comparable to the control in terms of distribution and intensity levels (**Figure R3.1a, c**). During mitosis, the CDK5RAP2 KO cell line showed a slight reduction in γ-tubulin levels at metaphase centrosomes, but the CDK5RAP2ΔCM1 cells seemed to be less affected (**Figure R3.1b, d**). Taken together with the observation from Chapter 2, where I used a different CDK5RAP2 knock out cell line with p53 WT background, it can be concluded that the presence of CDK5RAP2 is not essential for γTuRC recruitment to interphase centrosomes.

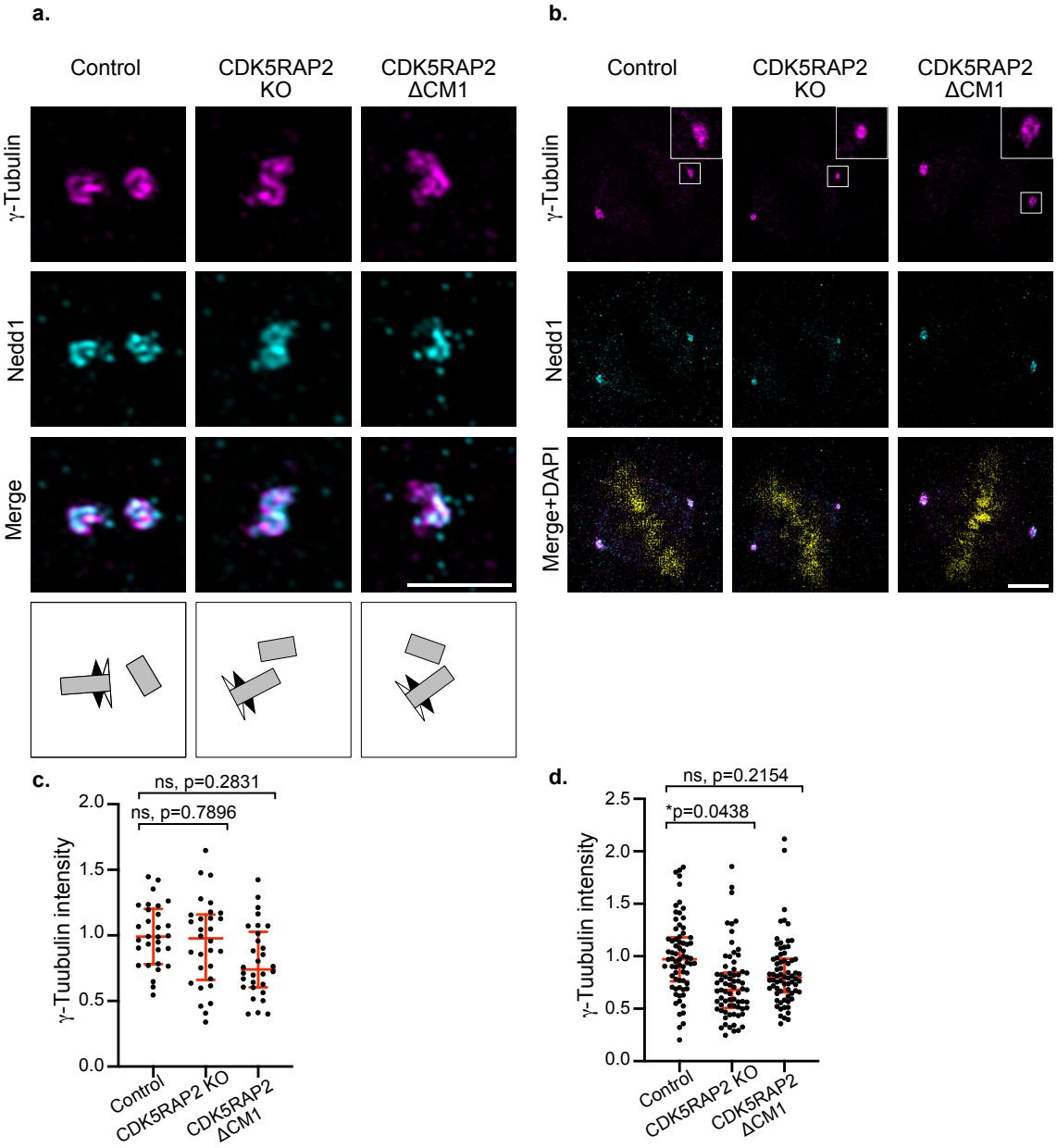


Figure R3.1: CM1 motif from CDK5RAP2 is not essential for γTuRC localization

Representative IF images of γ-tubulin centrosomal localization in RPE1 p53 KO control, CDK5RAP2 KO and CDK5RAP2ΔCM1 cells.
a. Interphase centrosome, scale bar 1µm. **b.** mitotic centrosomes, scale bar 5µm.
c. γ-Tubulin intensities as in (**a**) normalized to the average of control cells. Three independent experiments, 30 cells analyzed in each condition.
d. γ-Tubulin intensities as in (**b**) normalized to the average of control cells. Three independent experiments, 35 cells analyzed in each condition. Horizontal bars and whiskers indicate median and interquartile range respectively, p values as indicated, ns: not significant.

To see if centrosomal microtubule nucleation was disrupted in these cells, I performed short microtubule regrowth assays after cold depolymerization. The CDK5RAP2 KO and CDK5RAP2ΔCM1 cell lines managed to produce microtubule asters at the centrosome upon 3s of regrowth similar to the control cells (**Figure R3.2**). Although CDK5RAP2ΔCM1 cells appeared to have slightly weaker centrosomal asters, the difference was not significant.

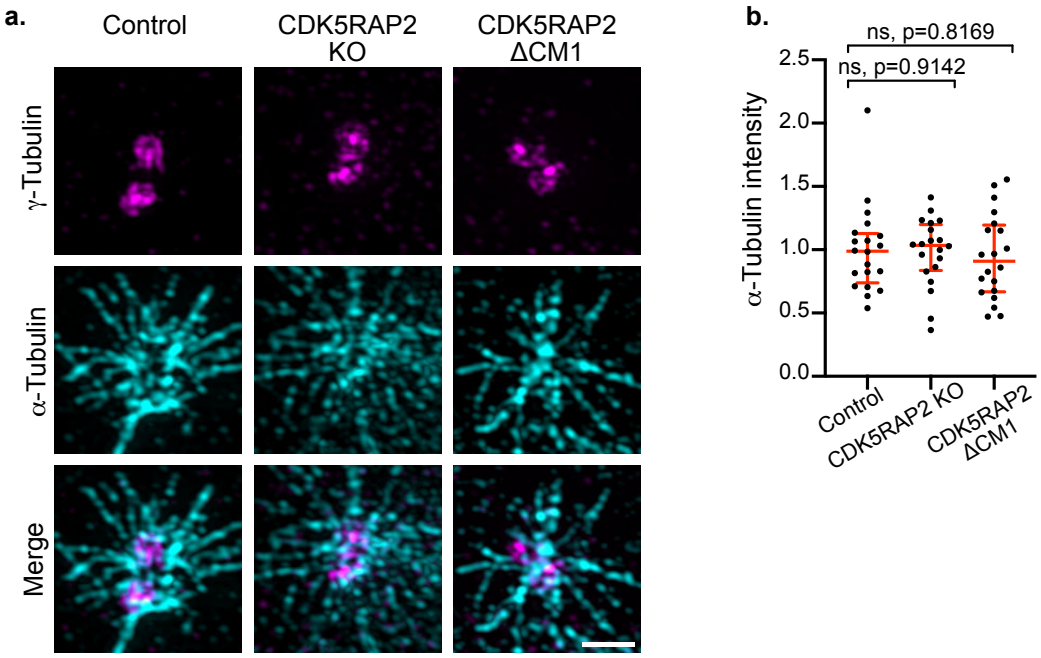


Figure R3.2: Loss of CM1 motif from CDK5RAP2 does not impair microtubule nucleation

a. RPE1 p53 KO control, CDK5RAP2 KO and CDK5RAP2ΔCM1 cells upon 3s of microtubule regrowth following cold depolymerization. Scale bar 1μm.

b. α-Tubulin intensities as in (**a**) normalized to the average of control cells. Two independent experiments, 20 cells analyzed in each condition. Horizontal bars and whiskers indicate median and interquartile range respectively, p values as indicated, ns: not significant.

GCP2 N-terminal region is required for γTuRC-CM1 interaction

So, far the results based on CDK5RAP2 KO and CDK5RAP2ΔCM1 suggested that the CM1 domain of CDK5RAP2 is not crucial for γTuRC recruitment and activation at the interphase centrosome. Apart from CDK5RAP2, Myomegalin (MMG) is another human protein that contain the CM1 domain and is implicated in centrosomal and Golgi associated nucleation (Roubin et al., 2013). Moreover, microtubule associated proteins like TPX2 have partial CM1 domain (Alfaro-Aco et al., 2017) and possibly there are additional proteins with CM1 or CM1-like domains that can perform similar functions.

Recent studies on the structure of human γTuRC complex have identified that the CM1 domain binds to the γTuRC via a specific module formed by the γTuRC component MZT2(GCP8) and the N-terminus of GCP2 that is located at the outer side of the complex (Wieczorek, Huang, et al., 2020; Zimmermann et al., 2020). To ensure inhibition of all CM1 interactions, we decide to delete the N-terminal region of GCP2, to generate cells, in which γTuRC would be insensitive to activation by any CM1. Here I use CRISPR edited, RPE1 p53 KO cells lines with either the whole N-terminus of GCP2 (corresponding to amino acids 1-216) deleted (referred to as GCP2ΔNTE) or cells that had exon 3 deleted, thereby removing only a segment within the N-terminus corresponding to amino acids 51-94 (GCP2Δexon3) (**Figure R3.3a**). These clonal lines were also generated by Fabian Zimmermann who verified their ability to integrate with other GCPs to assemble a γTuRC.

I tested for γTuRC-CM1 interaction in these cells by co-immunoprecipitation assay using a FLAG-CM1 construct. Control and GCP2 mutant cell lines transiently transfected with the FLAG-CM1 constructs were subjected to immunoprecipitation using anti-FLAG antibody-coupled sepharose beads. In the control cells, γTuRC was abundantly present in the pull-down as seen by probing for γ-tubulin, GCP2 and GCP6. In contrast, in the pull-downs from mutant cells γTuRC components were undetectable (**Figure R3.3b**). Therefore, I conclude that the mutant cells have lost the CM1-γTuRC interaction. This is further verified by the observation that upon overexpression of GFP tagged CM1 construct in the cytoplasm, the mutant cells failed to induce cytoplasmic microtubule nucleation as seen in the control cells in a regrowth assay (**Figure R3.3c, d**).

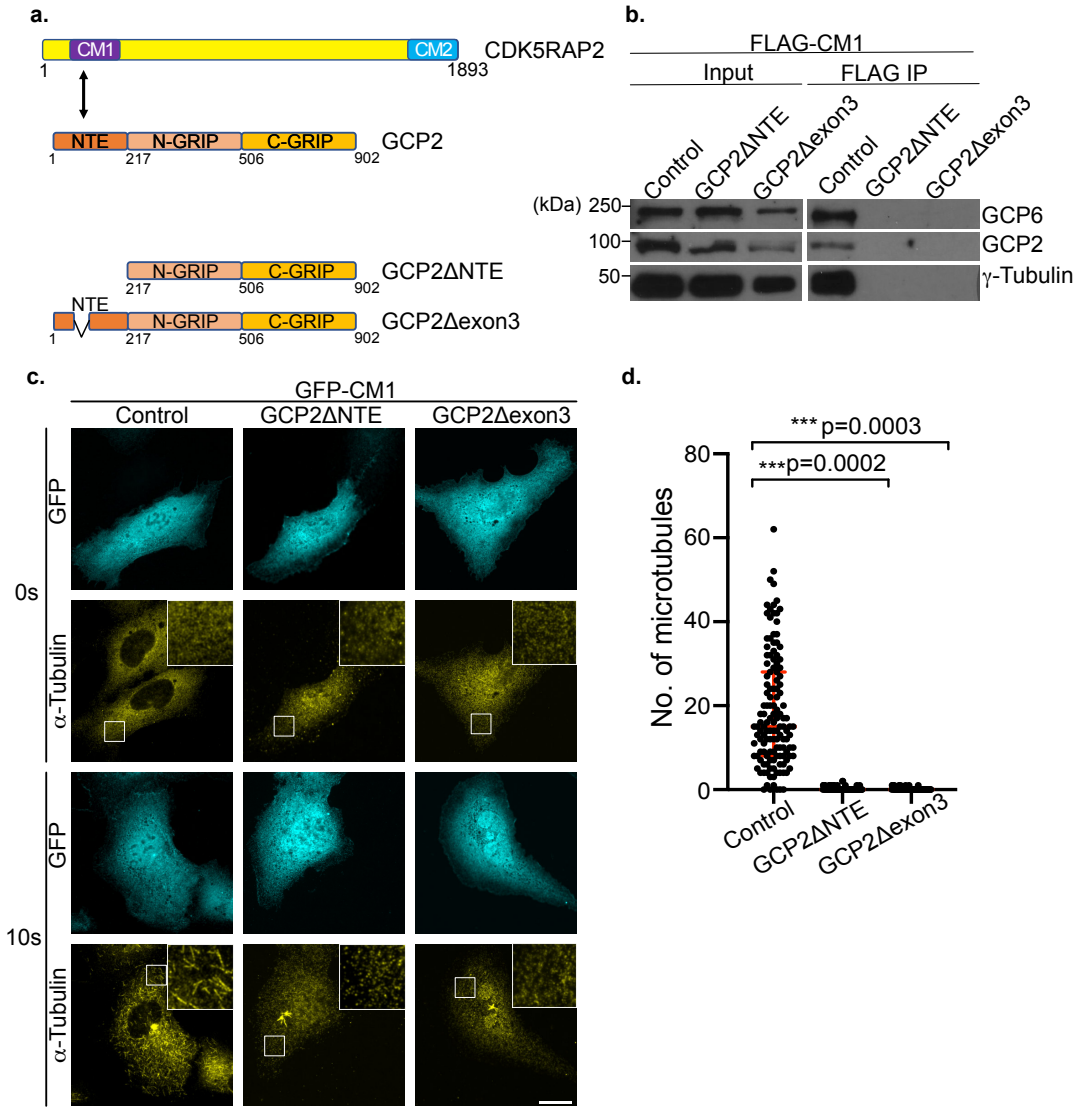


Figure R3.3: GCP2 N-terminal region is required for γTuRC-CM1 interaction

a. Schematic representation of the interacting regions between CDK5RAP2 and GCP2 and the genomic deletions generated for the GCP2 mutant cell lines

b. Lysates from RPE1 p53 control and GCP2 mutant cell lines transiently expressing FLAG-CM1 constructs were subjected to immunoprecipitation using anti-FLAG antibody conjugated sepharose beads and analyzed in a western blot by probing for the indicated γTuRC components.

c. Control and GCP2 mutant cell lines transfected with GFP-CM1 construct were subjected to microtubule regrowth assay and visualized using antibodies against GFP and α-tubulin. Scale bar 10μm.

d. Number of cytoplasmic microtubules in a fixed square ROI in the regrowth images as in (**c**) were counted in each of the cell line and plotted. 3 independent experiments, 90 cells analyzed in each condition. Horizontal bars and whiskers indicate median and interquartile range respectively, p values indicated in the graph.

GCP2ΔNTE and GCP2Δexon3 cells have strongly reduced centrosomal nucleation

While loss of endogenous CM1 domain in CDK5RAP2 or the whole of CDK5RAP2 protein did not affect interphase centrosomal microtubule nucleation significantly, short regrowth assays on the GCP2 mutant cell lines led to unexpected phenotypes.

Whereas the control cells showed intense microtubule asters around the centrosomes just after 3s of regrowth at 37ºC, the mutant cells had severe nucleation defects, with a majority of the cells having less than five microtubules growing out of the centrosomes (**Figure R3.4a**). Notably, the few microtubules formed in the GCP2 mutant cells were predominantly associated with the subdistal appendages further confirming the SDAs as a nucleation site at the centrosome. Moreover, a closer look at the control cells indicated that SDA associated nucleation is not a specific feature of the mutants, but also common in the control scenario.

GCP2ΔNTE cells fail to recruit γTuRC to the PCM

While observing the nucleation defects in the mutant cell lines, we noticed that the centrosomal γ-tubulin in these cells did not look comparable to the control. Whereas the GCP2Δexon3 cells showed a moderate reduction for γ-tubulin at the PCM compared to the control cells, in the GCP2ΔNTE cells γ-tubulin was strongly reduced by ~70%. Strikingly, the remaining centrosomal γ-tubulin signal was entirely restricted to the lumen and PCM-associated γ-tubulin was essentially undetectable (**Figure R3.4a, c**).

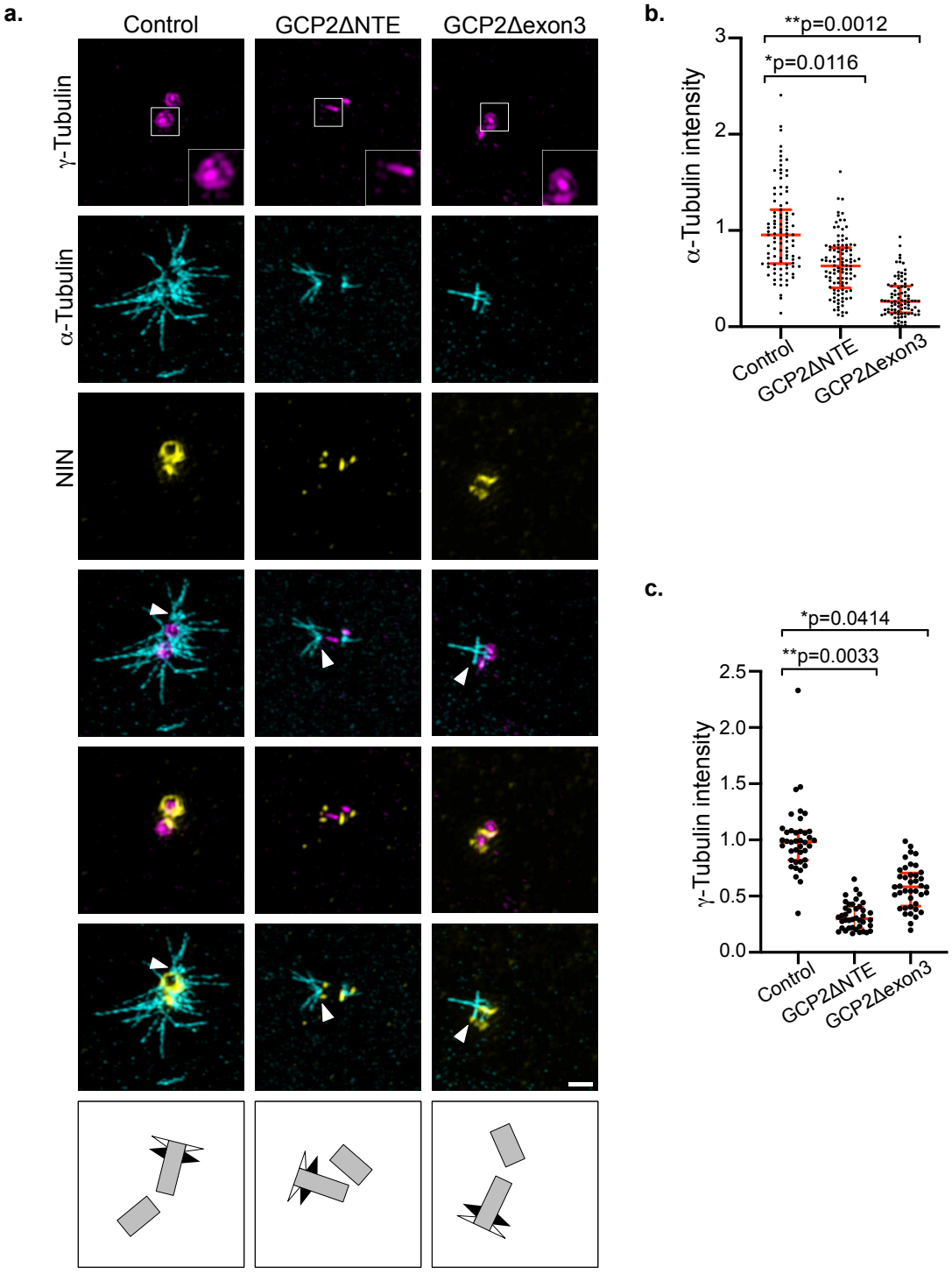


Figure R3.4: Loss of GCP2 N-terminus impairs centrosomal microtubule nucleation

a. Representative IF images of RPE1 p53 KO control and GCP2 mutant cells subjected to a short regrowth for 3s after cold depolymerization of microtubules. NIN and γ-tubulin mark the centrosome and newly formed microtubules are visualized by staining for α-tubulin. Centriole orientations are shown in the cartoon below each panel. Scale bar 1μm.

b. Intensity of centrosomal microtubule asters upon short regrowth in the control and GCP2 mutant cell lines as seen in (**a**) measured and plotted in the graph. Three independent experiments, 90 cells analyzed in each condition.

c. centrosomal γ-tubulin levels as seen in (**a**). Three independent experiments, 40 cells analyzed in each condition. Horizontal bars and whiskers indicate median and interquartile range respectively, p values as indicated in the graph.

GCP2 mutant cells have reduced Golgi nucleation

In RPE1 cells, Golgi plays an important role in nucleating and organizing microtubules. The strong nucleation defects at the centrosome in GCP2ΔNTE and GCP2Δexon3 cells prompted us to see if non-centrosomal MTOCs such as Golgi is also affected in these cells. I performed the same regrowth experiment for a slightly longer period of 10s to clearly visualize the Golgi derived microtubules as they do not form as quick as the centrosomal asters. At 10s, the centrosomal microtubules appeared longer in both control and GCP2 mutant cells. Both the mutants showed almost no microtubules associated with the Golgi, whereas the control cells displayed several short microtubules coming from this MTOC as seen by their association with the cis-Golgi marker GM130 (**Figure R3.5a**). The effect was quantified by counting the number of microtubules in each condition and the analysis revealed a significant reduction of Golgi derived microtubules in GCP2 mutant cells (**Figure R3.5b**).

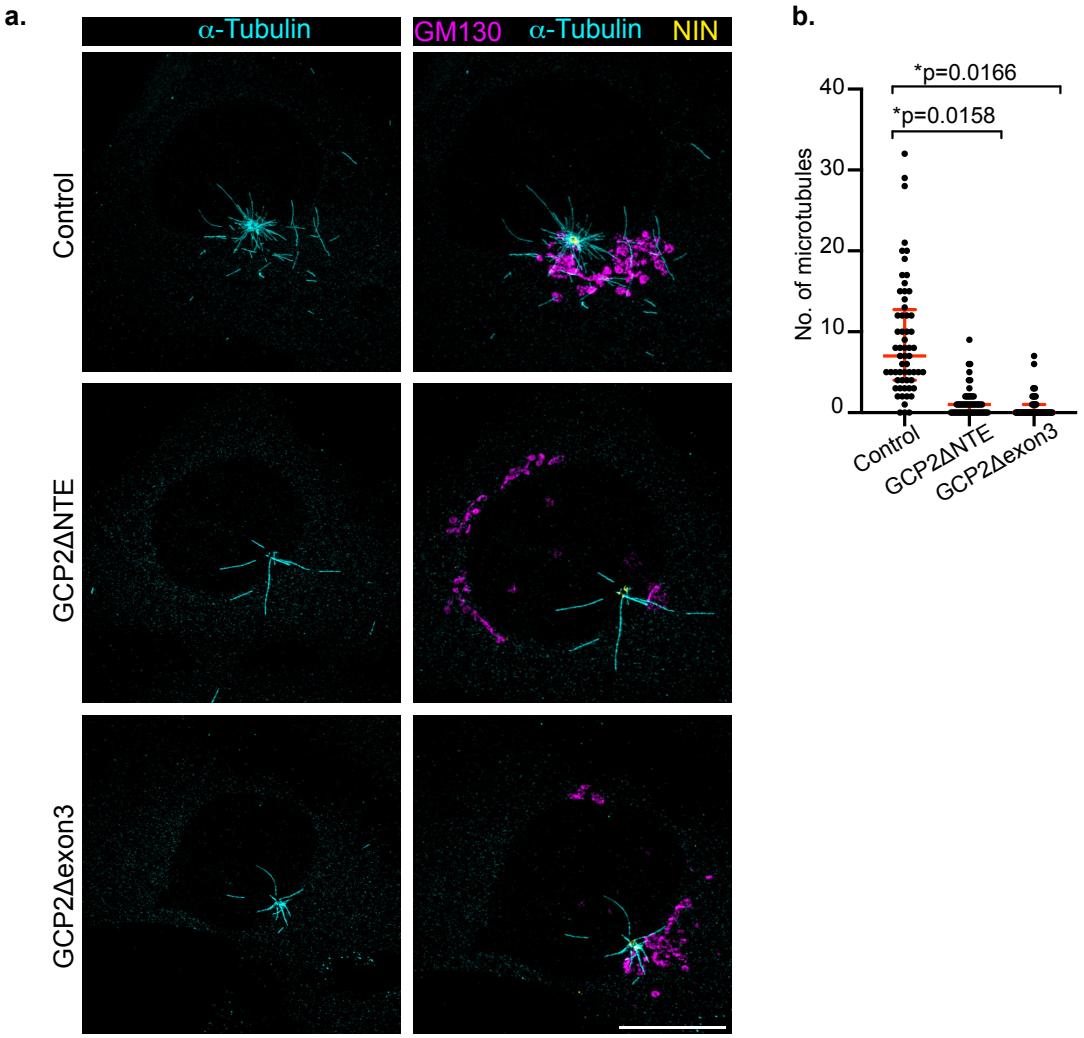


Figure R3.5: GCP2 mutants show reduced Golgi nucleation

a. Microtubules in RPE1 p53 KO control, GCP2ΔNTE and GCP2Δexon3 cells after cold depolymerization followed by regrowth at 37∫C for 10s, co-stained with Golgi marker GM130 and centrosomal marker NIN to see microtubules formed at either MTOCs. Scale bar 10μm.

b. Number of Golgi associated microtubules as seen in **(a)** counted and plotted. Three independent experiments, 60 cells analyzed in each condition. horizontal bars and whiskers indicate median and interquartile range respectively, p values as indicated in the graph.

GCP2 mutant cells show anchoring defects

At 10s of regrowth, while the GCP2 mutant cells had longer centrosomal microtubules, I noticed some of them were not directly attached to the centrosome or Golgi. Although the control cells also contained a couple of unattached short microtubules in the cytoplasm,

which probably resulted from slow inefficient nucleation at the cytoplasm, the longer microtubules in the mutant cells closer to the centrosome suggested the possibility that they were detached from the centrosome. To distinguish this better, I performed an even longer regrowth for 30s where I expected the centrosome derived microtubules to be longer than the ones formed by the slow nucleation in the cytoplasm.

Notably at this stage of regrowth, I could observe several longer microtubules in the mutant cells, some of them also coming from the Golgi, suggesting that given enough time these cells might overcome their low nucleation efficiency. On the other hand, while the control cells produced a long radial array at the centrosome, both mutants had a disorganized network with several unattached microtubules around the centrosome (**Figure R3.6a**, zoomed-in panels with arrowheads). To specifically account for microtubules that might have detached from the centrosome, I decided to count the number of microtubules not attached to either centrosome or Golgi that are at least 5μm long and within a radius of 25μm from the centrosome. While these numbers were chosen arbitrarily, it was based on the assumption that any microtubule nucleated in the cytoplasm will be much shorter due to their slow kinetics and be distributed anywhere in the cytoplasm, not necessarily close to the centrosome, while the centrosomal microtubules will be longer and be closer in proximity.

While there were some of the microtubules in the control cells satisfied the above criteria for being detached from the centrosome, the count was nearly double that in the GCP2ΔNTE cells suggesting a defect in anchoring microtubules at the centrosome after nucleation (**Figure R3.6b**). The GCP2Δexon3 cells had relatively fewer number of detached microtubules. This is a preliminary result and needs to be replicated and verified. Of note is that the mutant cells displayed NIN at the SDAs similar to the controls therefore any anchoring defect, if present, might be resulting from the inability of γTuRC to stably attach to the PCM and outer centriolar regions.

If there is a defect in microtubule anchoring at the centrosome, one would expect a loss of radial organization of interphase microtubule array in the steady state. To check this, I decided to check again the steady state microtubule network in the mutant cell lines.

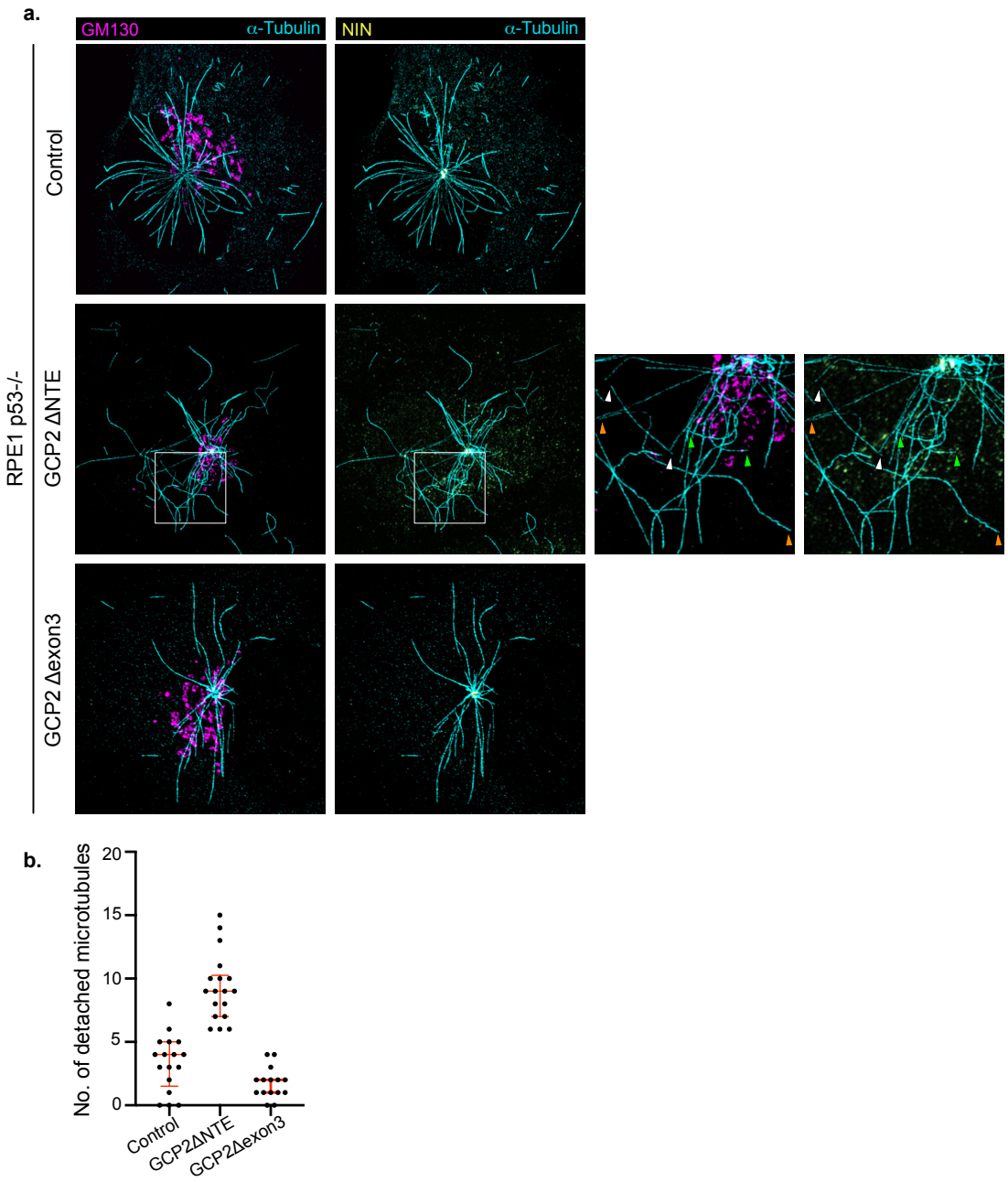


Figure R3.6: GCP2ΔNTE cells show anchoring defects

a. Microtubules in RPE1 p53 control and GCP2 mutant cells after cold depolymerization followed by regrowth for 30s, co-stained with Golgi marker GM130 and centrosomal marker NIN to see anchoring at either MTOCs. Unattached microtubules in GCP2NTE cells highlighted on either ends by an arrowhead of the same color in the zoomed-in panels on the right.

b. Number of microtubules that are at least 5µm long and are within a radius of 25µm from the centrosome from the images as in (**a**) were counted and plotted. One experiment, 15 cells analyzed in each condition.

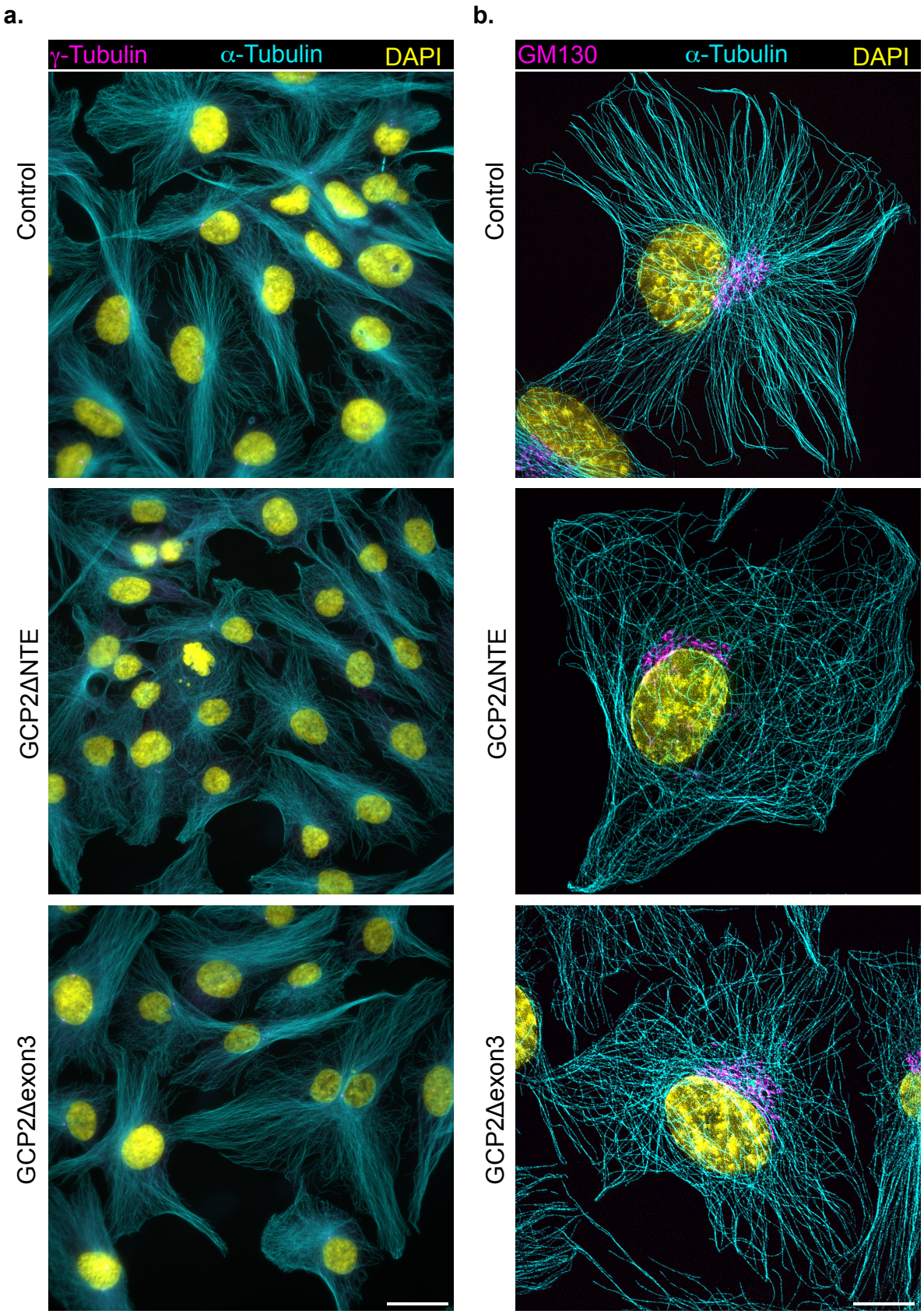

a.
γ-Tubulin
α-Tubulin
DAPI
Control
GCP2ΔNTE
GCP2Δexon3
b.
GM130
α-Tubulin
DAPI
Control
GCP2ΔNTE
GCP2Δexon3

Figure R3.7: Microtubule organization in GCP2 mutant cells lines

a. A wide field view of RPE1 p53 KO control and GCP2 mutant cells in the steady state without any treatments, stained with the indicated markers. Scale bar 20μm.

b. 3D-SIM image of a single cell from control and GCP2 mutant cells as in (**a**), stained with the indicated markers. Scale bar 20μm.

In RPE1 cells, while centrosome is still a major microtubule nucleator, at the steady state, the bulk of the microtubules are observed in association with the Golgi, forming an intense array. The GCP2ΔNTE cells showed a much weaker array of microtubules at the Golgi, which might result from a combination of slower nucleation dynamics and weaker anchoring (**Figure R3.7a**). In addition, the overall microtubule orientations appeared more disorganized in these cells compared to the uniform array in the control. By contrast, the GCP2Δexon3 cells did not show any noticeable defects in microtubule organization. A closer look using super resolution microscopy revealed some extreme cases of microtubule disorganization in the GCPΔNTE cells whereas the GCP2Δexon3 cells showed more organized microtubules, particularly at the Golgi (**Figure R3.7b**). These observations are preliminary and needs to be quantified further to know how generalized this phenotype is.

GCP2 mutant cells can nucleate microtubules in mitosis

The striking phenotype in microtubule nucleation and γ-tubulin recruitment in the GCP2ΔNTE and GCP2Δexon3 cells prompted us to check how the mitotic poles looked like in these cells, but to our surprise, the γ-tubulin in mitotic cells looked normal and had the appearance of an expanded PCM (**Figure R3.8a**). Upon quantification, these cells still showed lower γ-tubulin intensities compared to the control, but the difference was much less profound than in the interphase centrosomes (**Figure R3.8b**).

Looking at microtubule nucleation from mitotic centrosomes by a regrowth assay revealed that GCP2ΔNTE cells managed to nucleate microtubules to a similar extent to that of the control. While GCP2ΔEx.3 cells did show a reduced aster intensity, they still had a strong aster, compared to the very few numbers of microtubules seen in interphase cells (**Figure R3.8c**). A wider field view of GCP2ΔNTE shows how their ability to nucleate microtubules at the centrosome contrasts between interphase and mitosis (**Figure R3.8d**).

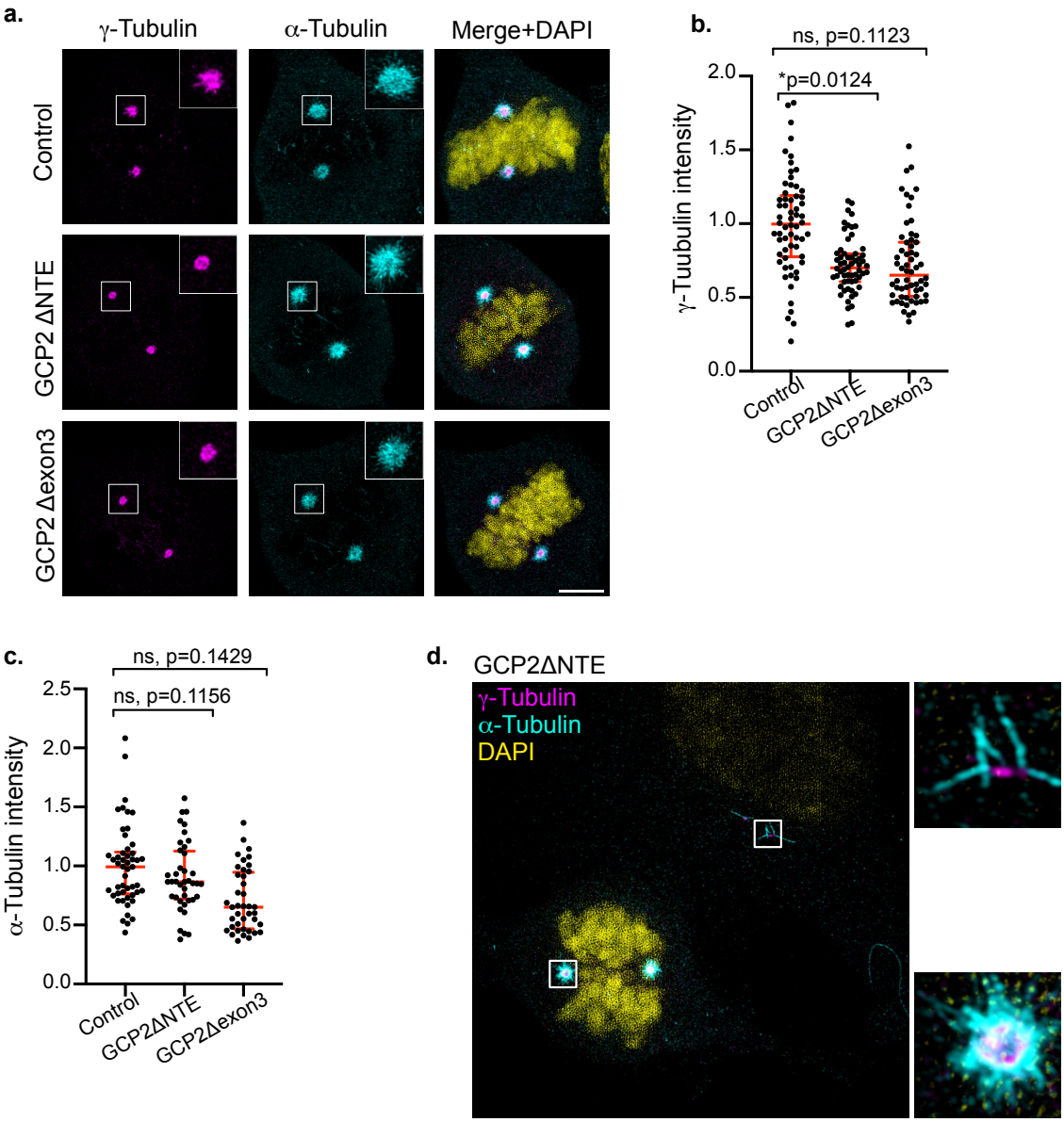


Figure R3.8: GCP2 mutants have normal centrosome function in mitosis

a. Mitotic cells from the control and GCP2 mutant cell lines subjected to microtubule regrowth assay. Scale bar 5μm.

b. centrosomal γ-tubulin levels as seen in (**a**). Three independent experiments, 30 cells analyzed in each condition.

c. Intensity of centrosomal microtubule asters upon short regrowth in the control and GCP2 mutant cell lines as see in (**a**) measured and plotted in the graph. Two independent experiments, 20 cells analyzed in each condition. Horizontal bars and whiskers indicate median and interquartile range respectively, p values as indicated in the graph, ns: not significant.

d. Wider field view of GCP2ΔNTE cells showing microtubule regrowth in neighboring interphase and mitotic cells.

GCP2ΔNTE cells have no alterations in the PCM structure

Considering the loss of γTuRC specifically at the PCM in the GCP2ΔNTE cell lines in the interphase, we wondered if the PCM itself is affected in these cells. To verify that I looked at the core PCM components Pericentrin (PCNT) and CDK5RAP2. Interestingly, in the mutant cells both these proteins maintained their centrosomal distribution similar to the controls as observed in the super resolution images (**Figure R3.9**).

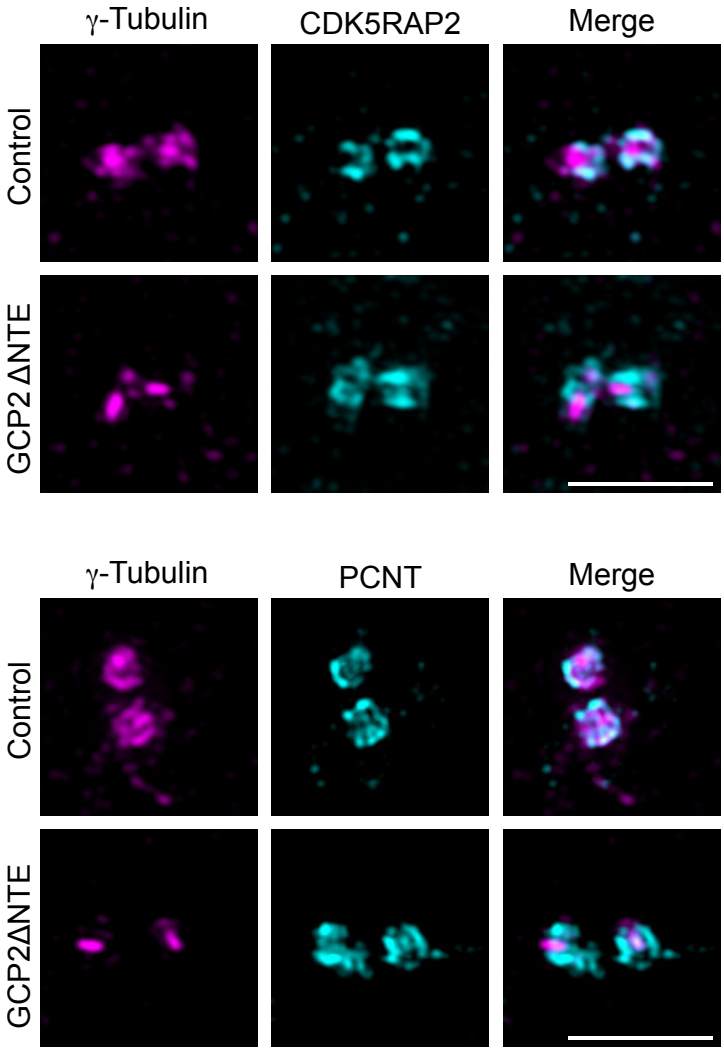


Figure R3.9: Core PCM structure is not altered in GCP2 ΔNTE cells

Representative IF images showing the localization of core PCM proteins CDK5RAP2 and Pericentrin (PCNT) in RPE1 p53 KO control vs GCP2 ΔNTE cells. Scale bar 1μm.

GCP2ΔNTE cells are defective in dynamic ch-TOG localization to the PCM

As the GCP2ΔNTE cells show a specific defect on γ-tubulin PCM localization in interphase but not in mitosis, we wondered whether this is connected to ch-TOG, as we have observed similar phenotypes in cells depleted of ch-TOG. I decided to check if ch-TOG behaves normally in these cells. As we know from chapter 2, ch-TOG has a dynamic localization at the centrosome. In steady state conditions, there is a stable pool of ch-TOG at the SDAs, but upon microtubule depolymerization it occupies a larger area at the PCM.

When I stained for ch-TOG in GCP2ΔNTE cells, I could observe the previously described localization at the SDAs in the presence of microtubules. Upon depolymerization of microtubules in the control cells ch-TOG appeared at the PCM, and the overall ch-TOG levels at the centrosome nearly doubled, but the GCP2ΔNTE cells showed no PCM associated ch-TOG, while the stable SDA-associated pool of ch-TOG remained (**Figure R3.10**). The overall ch-TOG levels at the centrosome in these cells remained unchanged after microtubule depolymerization, suggesting that dynamic localization of ch-TOG to the PCM is impaired in these cells.

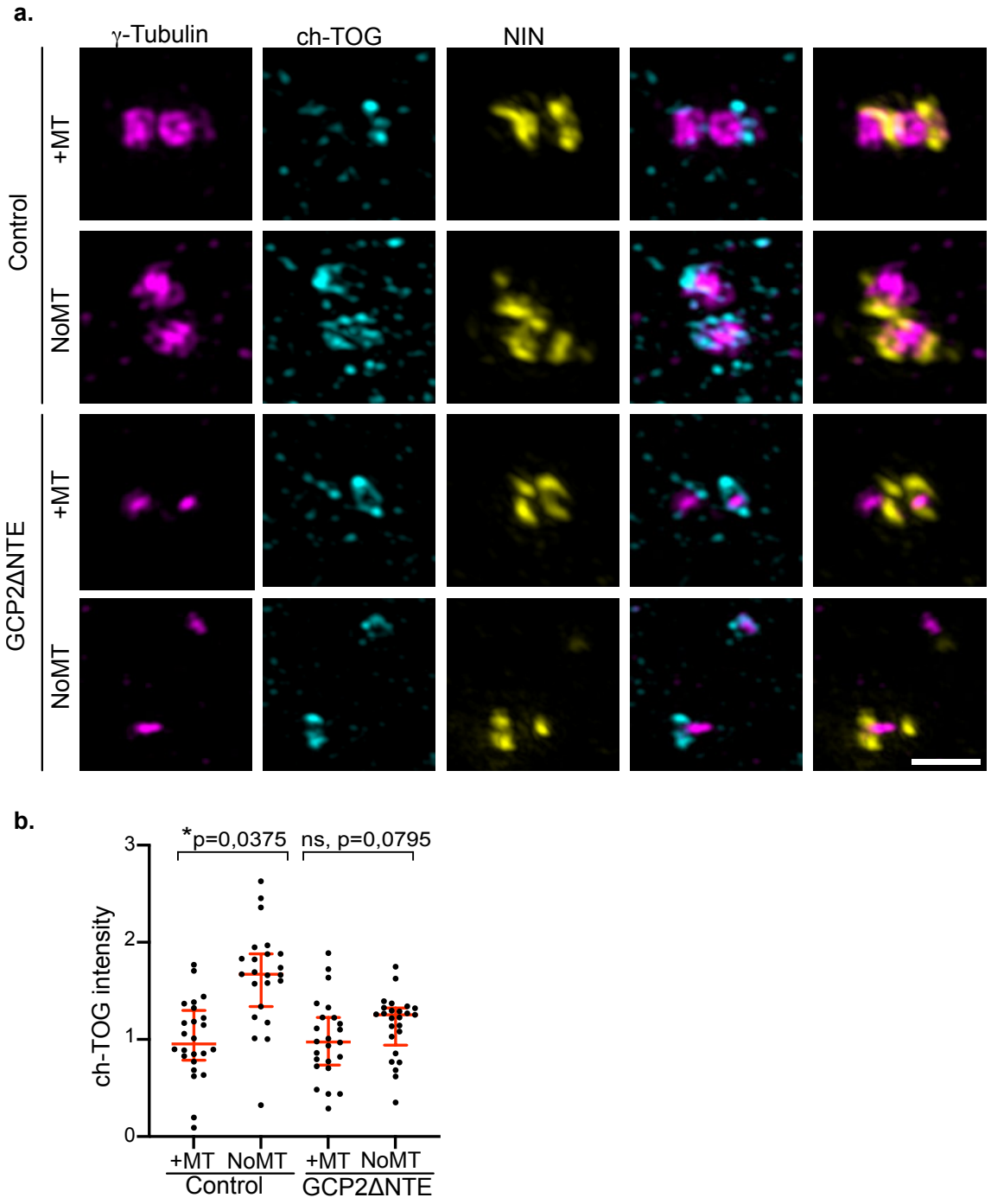


Figure R3.10: ch-TOG transient PCM localization is impaired in GCP2ΔNTE cells

a. ch-TOG centrosomal localization in RPE1 p53 control and GCP2 ΔNTE cells in the presence of microtubules (+MT) or after cold depolymerization (NoMT). Scale bar 1μm.
b. centrosomal ch-TOG intensities as in (**a**). Two independent experiments, 25 cells analyzed in each condition

ch-TOG enhances nucleation from cytoplasmic γTuRC-CM1 complexes

Binding of CM1 domain was shown to activate nucleation from γTuRC in vitro and in the cytoplasm. Human ch-TOG and its orthologue XMAP215 have also been implicated in nucleation activation in several *in vitro* studies and in cells as we have seen in chapter 2. The GCP2ΔNTE mutants show defect in CM1 mediated nucleation in the cytoplasm as well as in ch-TOG PCM localization. In addition, GCP2ΔNTE cells show defects in γ-tubulin PCM localization similar to ch-TOG depleted conditions. This suggested the possibility that the CM1 binding region in GCP2 might also mediate interaction with ch-TOG simultaneously. As CM1-mediated and ch-TOG-mediated mechanisms of microtubule nucleation have been studied independently in various systems, we wondered if these two factors contribute to microtubule nucleation by working together.

To check this idea, I decided to repeat the well-established experiment of cytoplasmic microtubule regrowth upon GCP-CM1 overexpression in cells depleted of ch-TOG. To our surprise, we could see a reduced number of microtubules formed in the ch-TOG depleted cells. Notably, in the cells that managed nucleation, only very short microtubules were visible, indicating slow or non-persistent growth (**Figure R3.11a**). Upon quantification, the difference in microtubule number appeared to be not significant, which might result from the high variation in the data distribution (**Figure R3.11b**). Nevertheless, we could observe a tendency of reduction in number and length of the cytoplasmic microtubules that suggest a potential role for ch-TOG in ectopic microtubule nucleation by CM1-bound γTuRCs, as well as in microtubule elongation, which is expected of a microtubule polymerase.

In summary, these results confirm that CDK5RAP2 association with γTuRC mediated by its CM1 domain is not a crucial factor for neither γTuRC centrosome recruitment nor centrosomal microtubule nucleation. The CM1 binding region within GCP2 is essential for microtubule nucleation a process that might involve other accessory factors that may or may not contain a CM1 domain. A larger region in the N-terminus mediates interactions necessary for γTuRC PCM recruitment in interphase, and possibly involve ch-TOG in this process. Moreover, ch-TOG plays an active role in microtubule nucleation even in the presence of excess of CM1 bound γTuRC.

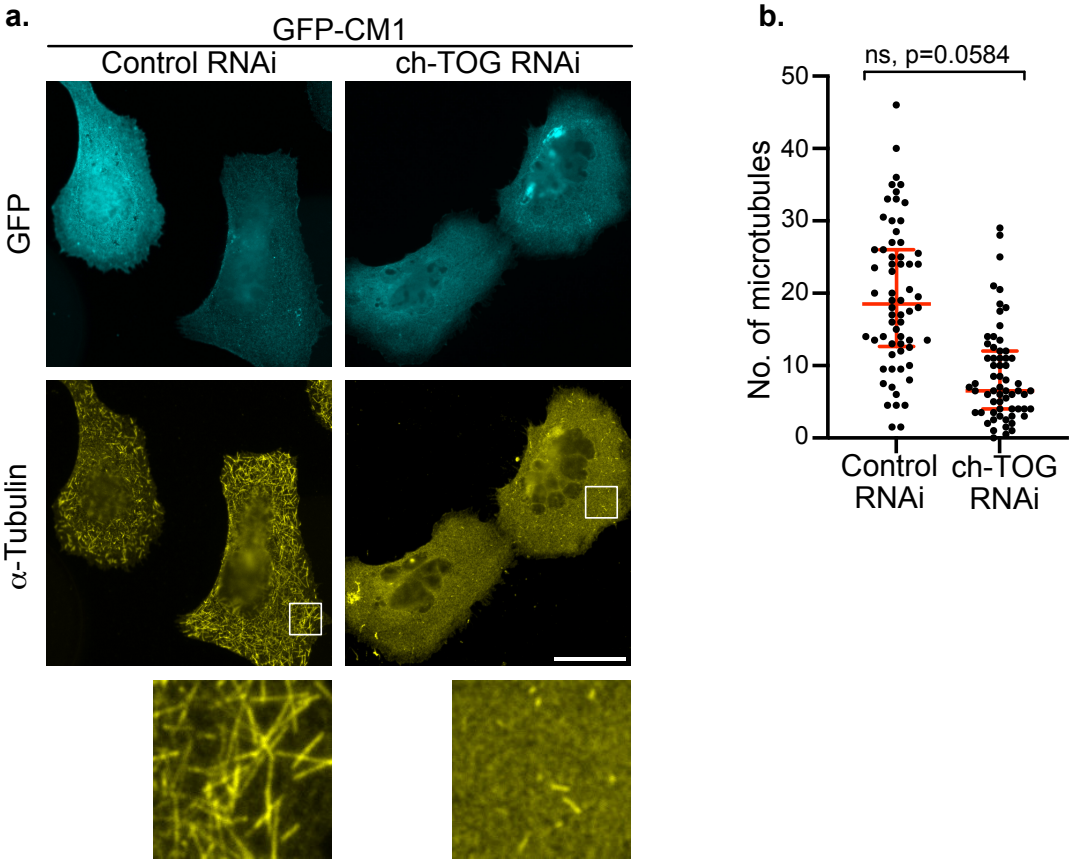


Figure R3.11: ch-TOG enhances microtubule nucleation from cytoplasmic γTuRC-CM1 complexes

a. U2OS cells treated with control or ch-TOG siRNA were transfected with GFP tagged CM1 construct and subjected to microtubule regrowth assay. Cells expressing the CM1 were visualized using antibody against the GFP tag and cytoplasmic microtubules were observed by staining for α-tubulin. Scale bar 10μm.

b. Number of microtubules in a defined square ROI in the cytoplasm, away from the centrosome were counted and plotted. Three independent experiments, 60 cells analyzed in each condition. Horizontal bars and whiskers indicate median and interquartile range respectively, p value as indicated in the graph, ns: not significant.

DISCUSSION

To control the organization of microtubule arrays in space and time, cells need to regulate the nucleation from γ-tubulin complexes. In budding yeast, nucleation is stimulated upon Spc72 and Spc110-mediated recruitment of γTuSCs to the SPBs where the complex undergoes oligomerization to become a ring-shaped template. The presence of preassembled rings of γ-tubulin complexes (γTuRCs) in the cytoplasm of higher eukaryotes creates the need for an additional activation step specifically at the MTOCs. The lack of spontaneous nucleation from the cytoplasm and the low nucleation efficiency of purified γTuRC *in vitro* suggested a role of the MTOC environment in promoting nucleation from these complexes. In this thesis I have analyzed three MTOC-associated factors that have proposed roles in γTuRC recruitment and stimulation of nucleation.

NINL is an SDA component that recruits essential MTOC components

Experiments from our lab as well as from the group of Erich Nigg have suggested a role of NINL in the formation and activity of the centrosomal MTOCs (Casenghi et al., 2003). Moreover, NINL overexpression resulted in the assembly of ectopic MTOCs in the cytoplasm. In my thesis I aimed at a functional analysis of NINL at the centrosome using knockdown and knockout approaches, which have not been attempted in previous work. We have observed no effect on centrosomal microtubule nucleation upon siRNA-mediated knock-down of NINL, however, the low efficacy of the siRNA treatments precludes us from completely ruling out NINL as a contributor in nucleation at the centrosome.

While it has been observed that removal of NINL through antibody microinjection results in reduced centrosomal nucleation specifically in interphase (Casenghi et al., 2003), the authors were not successful in testing this via a knock-down approach. Later studies observed mitotic defects upon siRNA-mediated knock-down of NINL (Jin et al., 2009; Yan et al., 2010), although the use of same set of siRNAs did not produce observable phenotypes in my experiments. Notably, the earlier observations have established a specific removal of NINL from the centrosome as the cells enter mitosis, therefore, it is counterintuitive that the depletion of NINL would have any impact in this stage of the cell cycle.

I also tried to generate a knock-out cell line using single and double guide-RNA strategies but was unsuccessful. The attempt to remove functional domains from the N-terminal genomic region using two guide RNAs did not produce homozygous modifications. The observation that NINL depletion results in severe defects in chromosome segregation and cytokinesis (Jin et al., 2009), might explain why generating a clonal line that has no functional NINL protein was unsuccessful. Moreover, the role of NINL as a dynein-dynactin adapter (Casenghi et al., 2005; Redwine et al., 2017) could also constitute an indispensable role for this protein in cells. Countering such an inference is a recent study that uses NINL knock-out cell lines to study anti-viral innate immune responses (Stevens et al., 2022), but curiously the authors did not describe any mitotic defects as observed in the knock-down studies.

Overall, these observations put forth conflicting reports regarding the centrosomal functions of NINL, therefore, it is crucial to test this more carefully. An interesting way to approach this would be to use conditional knock-down approaches. For example, an inducible degron-based system that allows quick degradation of the protein would be ideal to visualize the effects of NINL loss specifically during interphase without interfering with mitosis (Nabet et al., 2018).

On the other hand, testing the activity of NINL outside the centrosome revealed its ability in recruiting essential MTOC components. Based on the results from a previous student in the lab (Paz Domínguez, 2021), the NINL 1-287 region is sufficient to recruit γTuRC to ectopic sites as observed by a mitochondrial-targeting approach. By contrast, the larger NINL 1-442 fragment was required for microtubule nucleation at the ectopic site, suggesting that this region either activated γTuRC or recruited an activator. To identify a possible activator that is recruited specifically by NINL 1-442 region, I tested what additional factors are recruited to the mitochondrial surface by this region of NINL when targeted to this site. MAP7D3 and ch-TOG were the primary candidates as both these proteins were identified as proximal interactors of NINL in a BioID experiment performed by the previous student and have been implicated in promoting and/or stabilizing microtubule assembly (Sun et al., 2011; Yadav et al., 2014; Roostalu et al., 2015; Wieczorek et al., 2015).

Recruitment of MAP7D3 to the mitochondrial surface by NINL was observed with all the constructs tested including the smallest 1-287 construct, which lacks the ability to nucleate

microtubules at this site. The direct inference here would be that MAP7D3 is not sufficient to activate nucleation from γTuRC recruited by NINL 1-287. The question remains as to whether the presence of MAP7D3 at this site has a functional significance. Given the observed role of MAP7D3 in providing stability to microtubule assembly both *in vitro* and in cells (Sun et al., 2011; Yadav et al., 2014), NINL mediated recruitment of MAP7D3 to the mitochondria might contribute to the MTOC activity at this site despite not being sufficient to induce nucleation. Therefore, it is worth testing, using siRNA mediated knock-down of this protein, as to whether the presence of MAP7D3 contributes to microtubule nucleation or stabilization at this site.

Contrary to MAP7D3 recruitment, recruitment of ch-TOG by NINL 1-287 and NINL 1-442 correlated with their ability to nucleate microtubules at the mitochondrial surface. This suggests that ch-TOG is an important accessory factor for promoting nucleation from the γTuRC recruited by NINL at the mitochondrial surface. A role of ch-TOG in stimulating microtubule nucleation from γ-tubulin complexes was suggested by several studies that tested human ch-TOG as well as its orthologues such as *Xenopus* XMAP215, budding yeast Stu2 and fission yeast Alp14 (Popov et al., 2002; Flor-Parra et al., 2018; Gunzelmann et al., 2018; Thawani et al., 2018; Consolati et al., 2020). Our observations indicate that NINL plays an indirect role in microtubule nucleation by recruiting and bringing together the key players, γTuRC and ch-TOG. It needs to be noted that these finding are based on observations at an ectopic site that is artificially generated by our mitochondrial targeting system. Therefore, future work should address whether this role of NINL in facilitating the recruitment of factors needed for microtubule nucleation also extends to the centrosome.

The precise mapping of NINL localization at the centrosome using 3D-structured illumination microscopy (3D-SIM), revealed association with the SDAs as well as at the proximal ends of centrioles. However, the localization appeared to be much less defined at either of these sites compared to other SDA components such as NIN. Importantly, the extended proximal localization of the protein, particularly during early S phase might be a result of its functional role as a dynactin adapter, whereby NINL facilitates transport by the minus end directed motor. It is tempting to speculate that the increased levels of NINL at the centrioles in early S phase is an indication of increased rate of transport towards centrosome at this stage whereby essential components needed for centriole duplication are

recruited. But such an idea is a mere extrapolation from the observed NINL centrosomal accumulation and distribution and it remains to be verified whether NINL affects centriole duplication. Interesting to note here is that dynein-based transport plays a role in the accumulation of centriolar satellites during centriole duplication, which contributes to the increased supply of centrosomal proteins at this stage (Dammermann & Merdes, 2002; Kodani et al., 2015; Kubo et al., 1999).

In summary, our results suggest that NINL promotes microtubule nucleation through the recruitment of γTuRC, ch-TOG and possibly other factors. Further experiments that aim at a complete removal of NINL at the centrosome would be crucial to understanding how this ability of NINL contributes to centrosomal microtubule nucleation.

Interphase-specific role of ch-TOG in centrosomal γTuRC recruitment

Experiments that targeted NINL to the mitochondrial surface identified the involvement of ch-TOG in microtubule nucleation from this ectopic MTOC. On the other hand, at the centrosome, ch-TOG appears to be crucial for γTuRC recruitment as well. We have observed in our lab that ch-TOG depletion leads to a loss of γ-tubulin signals from the PCM corresponding to a ~50% reduction in fluorescence intensity (Ali et al., 2023). Strikingly, 3D SIM imaging revealed that the remaining signal is almost exclusively derived from the population of γ-tubulin in the centriole lumen, which is likely not involved in nucleation. In my experiments I have verified that such an effect is specific to interphase cells as γ-tubulin levels at mitotic centrosomes remained unperturbed upon ch-TOG depletion. In addition to the initial observation in U2OS cells made by Aamir Ali, a former postdoc in the lab, I was able to reproduce this result in RPE1 WT and CDK5RAP2 KO cells as well, suggesting a general role of ch-TOG in γTuRC recruitment to the interphase PCM that is not cell line-specific. As NINL 1-287 was able to recruit γTuRC to the mitochondrial surface without recruiting ch-TOG there, as seen in previous work (Paz Domínguez, 2021) and in **Figure R1.9**, it is possible that ch-TOG is not required for γTuRC localization at this site, however, this needs to be verified by checking if ch-TOG depletion affects γTuRC recruitment to mitochondria.

Interestingly, ch-TOG is not a constitutive PCM component, but is present only transiently at this site. Based on our regrowth experiments, ch-TOG localizes to the PCM upon microtubule depolymerization where it facilitates nucleation from the γTuRC, and once new microtubules start to form, the PCM bound ch-TOG gets displaced by its movement along the growing plus ends. This would mean that once microtubules are formed, further stable tethering of γTuRC at the PCM does not require ch-TOG. So, what exactly is the contribution of ch-TOG in γTuRC localization at the PCM? One possibility is that ch-TOG brings γTuRC to the PCM, where it gets anchored and nucleates microtubules, upon which, ch-TOG is free to move away along the new microtubules. If this is the case, such a targeting has to occur without the need for a stable interaction between the two as we do not see any detectable interaction in our experiments. Our co-IP experiments combined with the BioID pull down indicate that the interaction between ch-TOG and γTuRC is either transient or quite unstable. This interpretation is consistent with the transient localization of ch-TOG to the PCM, but invites questions as to what other factors contribute to the stable tethering of γTuRC there.

While the PCM scaffold protein CDK5RAP2 has been implicated in γTuRC recruitment to the centrosome similar to its budding yeast orthologues Spc72 and Spc110 (Knop & Schiebel, 1998; K.-W. Fong et al., 2008), later studies suggested this may not be a crucial factor (F. Chen et al., 2022; Gavilan et al., 2018). Our results from RPE1 CDK5RAP2 KO cells also confirm that CDK5RAP2 is not essential for γTuRC attachment to the centrosome in interphase, whereas ch-TOG is a major contributor in this process, possibly functioning along with other centrosomal adapters.

Another possible way by which ch-TOG facilitates γTuRC tethering at the centrosome is through its ability to stimulate nucleation. We see that upon ch-TOG depletion, centrosomal microtubule nucleation is drastically inhibited. It can be speculated that upon nucleation stimulated by ch-TOG, γTuRC becomes competent for binding to PCM adapters, which then allow stable tethering of the complex at the PCM. Such a mechanism would explain how ch-TOG could contribute to γTuRC localization at the PCM despite not being present there permanently. However, this raises several additional questions as to the identity of such a PCM-localized adapter that shows a specific preference for γTuRC after a nucleation event.

One candidate is CEP192, which has been shown to be required for the recruitment of γTuRC and NEDD1 to the PCM and centriole walls (Gavilan et al., 2018; Schweizer et al., 2021). However, there is no experimental evidence so far as to whether CEP192 shows differential binding to γTuRC before and after nucleation. In addition, if binding in nucleation-dependent, further exploration is required to understand what features of the γTuRC allow this distinction. A possible mechanism would be structural changes in the γTuRC such as closure of the ring after it has nucleated a microtubule.

Notably, any structural changes in γTuRC could be caused directly by ch-TOG association or be an indirect consequence of ch-TOG promoted nucleation, upon which the growing microtubule forces a matching symmetry in γTuRC, which then promotes adapter binding. Testing these hypotheses will involve answering the following questions: 1) does γTuRC show any changes in conformation before and after nucleation, 2) does CEP192 show a preference between free vs microtubule-bound γTuRC, 3) is there any other adapter at the PCM that behaves in this manner? A detailed structural analysis comparing the free γTuRC with γTuRC in a microtubule-bound state, combined with *in vitro* binding studies to check for differential binding of the proposed adapters in each of these conditions would be crucial in testing the role for such a mechanism. Additionally, the use of techniques like disulfide crosslinking to promote a closed conformation of the complex, as described in the case of budding yeast γTuSC oligomer (Kollman et al., 2015),would provide an opportunity to test binding preference of adapters towards open vs closed γTuRC *in vitro*.

However, before one delves into such complicated experiments, it is important to identify which PCM-associated adpaters may cooperate with ch-TOG to stably bind γTuRC. The first step would be depleting possible candidates including CEP192 to see which of them recapitulate the ch-TOG depletion phenotypes, which is expected if they function in the same pathway as ch-TOG. And once such candidates are identified, testing their interaction with γTuRC in control vs ch-TOG depleted cells using co-IPs would be important to verify a role of ch-TOG transient association with γTuRC in promoting γTuRC interaction with the PCM adapter. In the latter case, it would be ideal if a complete loss of ch-TOG in the test sample can be ensured using tools such as inducible knock-outs.

While we describe a transient association of ch-TOG with γTuRC to be essential for both γTuRC recruitment and microtubule nucleation, the exact nature of this interaction needs to be further explored. Observations from *Xenopus* XMAP215 suggests an interaction mediated via the C-terminus of the protein with γ-tubulin (Thawani et al., 2018). The same study also identified that whereas the C-terminus of XMAP215 is dispensable for its polymerase activity, this domain along with the TOG domains is essential for efficient microtubule nucleation in *Xenopus* egg extracts as well as from purified γTuRCs *in vitro*. Analysis of human ch-TOG in our lab also revealed the need for the C-terminus domain for its centrosomal localization, while the presence of the full length protein was required to recruit γTuRC to the PCM and to promote microtubule nucleation (Ali et al., 2023). Together these observations suggest that the polymerase activity of the N-terminal TOG domains coupled with the γTuRC interaction via the C-terminus is important for microtubule nucleation activity of ch-TOG. Possibly, the complete microtubule nucleation ability is also what facilitates γTuRC tethering at the PCM as we hypothesized above.

Notably, the lack of observable interaction between γTuRC and ch-TOG might result from the ability of ch-TOG to move towards the microtubule plus-ends upon stimulating nucleation as we have seen in **Figure R2.1**. Therefore, it is worth testing that if the C-terminal domain that lacks any polymerase activity can be seen to be stably interacting with the γTuRC. Furthermore, it is important to define more precisely the region in the ch-TOG C-terminus that mediates interaction with γTuRC. This would allow more specific disruption of the interaction.

Another open question here is how ch-TOG promotes nucleation from γTuRC. On a growing microtubule, ch-TOG progressively associates with the plus end where it promotes the addition of new tubulin dimers (**Figure I2.1**). A similar mechanism might be involved in the initial nucleation process as well. The unique structure of ch-TOG with linearly arranged TOG domains can potentially act as an extended "arm" that captures and brings tubulin dimers to the nucleator complex, thereby increasing the probability of tubulin binding to γTuRC.

Interestingly, combining the first two TOG domains with a microtubule binding region was not sufficient to stimulate nucleation in *Xenopus* egg extracts (Thawani et al., 2018) further verifying the need for the C-terminus. Constructs that contain the first two TOG domains connected to a microtubule binding region or to the C-terminus have been shown to have moderate polymerase activity based on *Xenopus* and *Drosophila* orthologues (Widlund et al., 2011; Byrnes & Slep, 2017; Thawani et al., 2018). Therefore, it would be interesting to see if such a moderate polymerase activity combined with γTuRC binding ability of the C-terminus would promote nucleation *in vitro* or in cells. If it does, the next step would be to check if such a construct containing TOG1, TOG2 and the C-terminus can also rescue the γTuRC localization defects seen when the endogenous ch-TOG is depleted. Such an experiment would be an important tool to check our hypothesis regarding the contribution from the nucleation activity of ch-TOG in stable tethering of γTuRC at the PCM. Moreover, if one identifies the exact region within the ch-TOG C-terminus that facilitate interaction with γTuRC as discussed earlier, and combines it with essential polymerization domains, one may be able to narrow down on the essential features required to stimulate nucleation from γTuRC.

XMAP215 orthologues have also been implicated in promoting the stability of nucleation intermediates in addition to their potential role in enhanced supply of tubulin dimers to the nucleator complex (Roostalu et al., 2015; Byrnes & Slep, 2017; Roostalu & Surrey, 2017). At the γTuRC, binding of new tubulin dimers may be followed by their dissociation unless a critical size or nucleus of tubulin polymer is reached, making the nucleation process inefficient. γTuRC is proposed to reduce this kinetic barrier in microtubule nucleation by providing a template for tubulin assembly, but due to its asymmetric structure γTuRC alone may not be sufficient to provide this function.

A model where a critical nucleus formed from 4 tubulin dimers on the neighboring positions on a γTuRC has been proposed recently based on biochemical and computational studies using *Xenopus* γTuRC (Thawani et al., 2020). A scenario where multiple ch-TOG molecules interact with a γTuRC and simultaneously bring multiple tubulin dimers to the complex would promote the lateral interactions between the newly added subunits, thereby reducing the rate of dissociation. Furthermore, it is interesting to hypothesize that a linear arrangement

of TOG domains might provide an additional physical support to the early nucleation intermediates to stabilize a nucleus until growth dominates. Such a mechanism would explain how ch-TOG is able to stimulate nucleation even in the absence of γTuRC, but the process is several folds more efficient when both work in combination (Roostalu et al., 2015; Thawani et al., 2018; King et al., 2020).

ch-TOG in microtubule nucleation at centrosomal and non-centrosomal sites

Our results establish ch-TOG as an essential factor in stimulating microtubule nucleation at the interphase centrosome. In addition, I also observed a decrease in the number of microtubules formed at the Golgi upon ch-TOG depletion in RPE1 cells. Given that the Golgi is a prominent non-centrosomal MTOC also in several differentiated cell types, this result suggest that ch-TOG may not only stimulate microtubule nucleation from the centrosome, but more broadly also from other MTOCs. Although ch-TOG signals were detected at the ends of microtubules growing from the Golgi, it was difficult to distinguish Golgi and microtubule associated ch-TOG signals from dispersed cytoplasmic signals. Therefore, I could not determine with confidence whether ch-TOG accumulates at the Golgi transiently like at the PCM.

Notably, an effect of ch-TOG depletion in microtubule nucleation from mitotic centrosomes has not been directly tested here. While normal levels of γ-tubulin at the mitotic centrosomes after ch-TOG depletion would indicate a normal level of nucleation at this stage, it needs to be verified.

ch-TOG at the SDAs

Another interesting observation is the presence of a stable pool of ch-TOG at the subdistal appendages. The observed loss of this pool of ch-TOG upon depletion of the inner SDA component CEP128 further confirms the localization being specific to the SDAs. Notably, the transient pool of ch-TOG that appear upon microtubule depolymerization was still

observable in CEP128 depleted cells, suggesting this localization is independent of ch-TOG's SDA localization.

We have seen that the ch-TOG that accumulates upon microtubule depolymerization is dispersed by its association with the newly formed microtubules during early regrowth conditions, correlating with its role in promoting nucleation (**Figure R2.1**). Interestingly, the transient association of ch-TOG was not only to the PCM but was present also at the distal centriolar regions, consistent with our idea that microtubule nucleation at the centrosome is not limited to the PCM. However, an additional stable pool of ch-TOG at the SDAs warrants the question if it performs functions other than its role in nucleation at this site. Given the role of SDAs in microtubule anchoring, ch-TOG may contribute to this function, possibly along with the established anchoring factor NIN, which would require a stable association with the anchoring site. Therefore, it needs to be tested whether ch-TOG depletion causes any anchoring defect at the centrosome and whether ch-TOG interacts with SDA proteins such as NIN. If any anchoring defects are observed, it is also important to check whether it can be rescued by expression of ch-TOG C-terminus, as the C-terminus alone was able to localize to the SDAs (Ali et al., 2023).

CM1 domain of CDK5RAP2 in γTuRC recruitment

As we have already discussed above, the association between γTuRC and CDK5RAP2 is not essential for its centrosomal recruitment in interphase unlike the situation with its orthologues from budding yeast, *Drosophila* or *C. elegans*. Further confirmation for this observation was made using a different CDK5RAP2 KO generated in our lab as well as a CDK5RAP2ΔCM1 cell line that has the γTuRC interacting domain removed. Observations from these cell lines also indicate that the CDK5RAP2-CM1 mediated activation of γTuRC is not a major nucleation pathway at the interphase centrosomes as both these cell lines managed to produce microtubules in a regrowth assay similar to the control.

Notably, the CDK5RAP2 KO cells displayed a reduction in the γ-tubulin levels at the mitotic centrosomes. The zoomed-in panels from **Figure R3.1** clearly demonstrate that these cells do have PCM-bound γ-tubulin, but the PCM itself appears smaller. This suggest that a lack of proper mitotic PCM expansion may cause the overall lower levels of γ-tubulin compared

to the control cells. On the other hand, the CDK5RAP2ΔCM1 cells did not show a significant reduction in γ-tubulin levels and displayed a PCM size similar to the control indicating that these cells have undergone PCM expansion at the onset of mitosis. Together these observations suggest that even at the mitotic centrosomes, the CM1 mediated interaction between CDK5RAP2 and γTuRC is not essential for γTuRC recruitment to the PCM. This is consistent with the recent observations that in the presence of centrioles both CDK5RAP2 and PCNT are dispensable for mitotic spindle assembly (Watanabe et al., 2020). The major function of CDK5RAP2 would be to act as a scaffold that allows expansion of the PCM and thus provide larger surface area for γ-tubulin docking, which becomes crucial when centrioles are perturbed (Watanabe et al., 2020). Notably, the relatively normal centrosomal γ-tubulin levels in CDK5RAP2ΔCM1 cells suggests additional binding regions or partners. Given the extensive phosphorylation of PCM proteins including CDK5RAP2 and PCNT at the onset of mitosis, it can be speculated that several additional interaction sites become active. Recent reports from human CDK5RAP2 and *Drosophila* Cnn identifies a role for phosphorylation in enabling CM1 binding to γTuRC (Tovey et al., 2021; Yang et al., 2023). Therefore, it would be interesting to see if mitotic phosphorylations of CDK5RAP2 reveal additional γTuRC binding sites in this protein.

CM1 binding region in γTuRC has broader functions

In contrast to the observations from CDK5RAP2 KO and CDK5RAP2ΔCM1 cells, removal of the CM1 binding site at the γTuRC through mutation of GCP2 resulted in a much stronger interphase defect. Both GCP2ΔNTE cells and GCP2Δexon3 cells showed severe nucleation defects in interphase with lower γ-tubulin levels at the centrosome.

As the phenotypes seen in GCP2 mutant cells are not shared by CDK5RAP2 KO or CDK5RAP2ΔCM1 cells, it can be concluded that the GCP2 N-terminal deletions affect not only CDK5RAP2 binding, but possibly other interactions as well. The pull downs and ectopic nucleation assays in CM1 overexpressed cells as seen in **Figure R3.3** clearly demonstrate that GCP2 N-terminal deletions abolish CM1 interaction and associated stimulation of nucleation. Therefore, one immediate question would be whether there exist proteins other than CDK5RAP2 that make use of a CM1 to bind γTuRC. In humans, the

CDK5RAP2 paralogue Myomegalin (MMG), has been described to contain a CM1 domain and to promote nucleation from interphase centrosomes as well as from the cis-Golgi (Roubin et al., 2013; Z. Wang et al., 2014). Since later studies suggested myomegalin is not crucial for centrosomal nucleation (Gavilan et al., 2018), this requires proper validation.

While it remains possible that myomegalin contributes to centrosomal γTuRC recruitment and activation, the N-terminal region in GCP2 may also be utilized for non-CM1 mediated interactions. Therefore, further dissection of this region to identify ideally point mutations that would specifically disrupt interactions with CM1 would be crucial to distinguish the effects resulting from different γTuRC interacting partners.

Interestingly, a non-CM1 mediated interaction at this region of GCP2 was recently described for KIF2A, facilitated by the MZT2 subunit (Shankar et al., 2022). The N-terminus of GCP2 has been shown to form a complex with the γTuRC subunit MZT2 (Wieczorek, Huang, et al., 2020; Zimmermann et al., 2020) and to promote γTuRC recruitment and microtubule nucleation at the centrosomes in interphase (Teixidó-Travesa et al., 2010). Therefore, it is important to analyze the contributions from MZT2 in the context of the phenotypes observed in GCP2 mutant cell lines.

While both the cell lines showed highly reduced γ-tubulin levels and microtubule nucleation at the interphase centrosomes, the complete loss of any PCM-associated γ-tubulin signal in the GCP2ΔNTE cells was quite striking. Although clonal variations might contribute to such differences, it is worth exploring whether the larger deletion may result in additional functional defects in this cell line compared to the GCP2Δexon3 cells. For example, the deletion of the GCP2 exon3 region described in this thesis has been shown to disrupt MZT2 binding in a separate study (Shankar et al., 2022). Thus, it is important to determine whether GCP2Δexon3 and GCP2ΔNTE cells differ in the severity by which interaction with γTuRC and other binding partners is disrupted.

Interesting to note is that both GCP2ΔNTE and GCP2Δexon3 cells showed strong centrosomal asters and higher levels of PCM-associated γ-tubulin during mitosis. This suggests that any interaction via the GCP2 N-terminus, whether or not CM1-mediated, is

not essential for nucleation activation from γTuRC during mitosis. Although the GCP2ΔNTE cells showed a slight reduction in γ-tubulin intensity at this stage, the presence of PCM-associated γ-tubulin was clearly visible in these images unlike in the case of interphase cells. Such an interphase-specific loss of γ-tubulin at the PCM closely resembled the phenotype from ch-TOG depleted cells, prompting me to look at ch-TOG localization in these cells.

Interestingly, GCP2ΔNTE cells also showed a reduction in ch-TOG accumulation at the interphase PCM upon microtubule depolymerization, suggesting ch-TOG transient PCM recruitment requires the N-terminal region of GCP2. It is tempting to speculate that ch-TOG transient interaction with γTuRC is mediated through this region in the GCP2 N-terminus, which would explain both the nucleation defect as well as the lack of PCM recruitment of γTuRC in interphase. Further experiments need to be done to verify this idea. As an interaction between ch-TOG and γTuRC was not observed in regular co-IPs, a BioID experiment using BirA-GCP3 as bait, which served to identify transient interaction between ch-TOG and γTuRC in control cells (**Figure R2.5**), could be performed in the mutant cells.

Although ch-TOG may contribute to the γ-tubulin localization defect seen in the GCP2 mutant cells, additional PCM-associated adapters might be required for stable integration of γTuRC here as we discussed in the previous section. Therefore, similar to the KIF2A interaction (Shankar et al., 2022), more centrosomal proteins might make use of the GCP2 N-terminus for binding to γTuRC, possibly facilitated by the binding of MZT2 to this region.

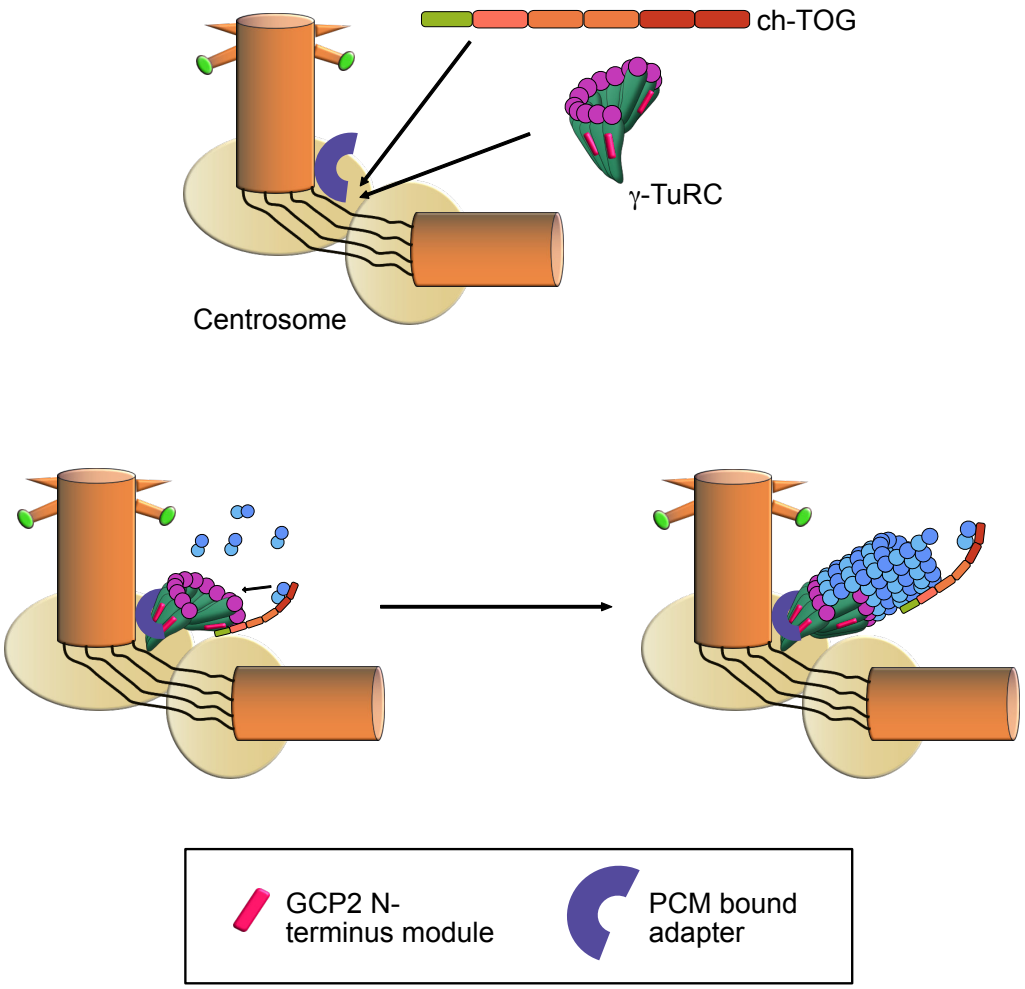


Figure D1: Model for γTuRC PCM localization

A model in which, ch-TOG and γTuRC interact transiently upon recruitment to the PCM, facilitating nucleation and γTuRC binding to the adapter. Following nucleation, ch-TOG leaves the PCM along with the growing end of the microtubule. The GCP2 N-terminus module mediates association of γTuRC with ch-TOG and also with the PCM adapter.

Two plausible candidates for interactions via GCP2 N-terminus are CEP192 and NIN. A role of GCP2 N-terminus in mediating γTuRC-NIN interaction might be relevant considering the anchoring defects seen in GCP2ΔNTE cells. While it is not clear if GCP2 N-terminus mediates γTuRC-NIN interaction, the N-terminus of NIN has been identified to be required for this association (Delgehyr et al., 2005). Moreover, perturbing this interaction at the centrosome by overexpressing the NIN C-terminus that displaces the endogenous protein has been reported to cause reduced centrosomal γ-tubulin, delayed

nucleation, and anchoring defects (Delgehyr et al., 2005). It is important to confirm that the reduction in γ-tubulin results from a specific loss of γ-tubulin at the PCM.

On the other hand, CEP192 depletion resulted in a specific removal of γ-tubulin and NEDD1 from the PCM and outer centriolar regions (Schweizer et al., 2021) and heavily reduced centrosomal nucleation in interphase (O'Rourke et al., 2014; Gavilan et al., 2018), phenotypes also observed for the GCP2 mutant lines. These observations may indicate that CEP192 affects γTuRC recruitment and stimulation of nucleation through interaction with the GCP2 N-terminus. Interestingly, mutations that perturb the Plk1 mediated phosphorylation of *Xenopus* Cep192 have been shown to disrupt its association with γTuRC and XMAP215 in mitotic extracts (Joukov et al., 2014), however it is not clear if similar interactions are promoted in interphase as well. While the mitosis-specific γTuRC binding sites identified in the *Xenopus* orthologue are not conserved in the human CEP192, it is possible that mitotic phosphorylation activates several additional interacting sites that can recruit γTuRC. Overall these observations recommend testing whether γTuRC directly associates with CEP192 in interphase, whether the GCP2 N-terminus is required to mediate such an interaction and whether ch-TOG has any influence on it.

Testing γTuRC interaction with NIN and CEP192 by co-IP in control and GCP2 mutant cell lines is necessary to test the ideas discussed above. Moreover, it would be interesting to see if there is any difference in interactions between GCP2ΔNTE and GCP2Δexon3 that will deepen our understanding of the exact binding sites of these partners on GCP2. If these candidates show a loss of interaction in the mutant cells compared to the controls, it is important to further test if such differences are also seen in wild type cells upon ch-TOG depletion similar to what discussed in the previous section. If yes, that would validate the hypothesis that ch-TOG transient association with γTuRC facilitates γTuRC binding to the PCM adapters and will reveal a role of GCP2 N-terminus in mediating these interactions. It would be interesting to expand these analyses to check for any effects from mitotic phosphorylations to explain why the effects we describe here are specific to interphase.

Important to note is that, here I have described a loss of ch-TOG transient localization to the PCM upon microtubule depolymerization only in GCP2ΔNTE cells. This needs to be

also tested in GCP2Δexon3 cells to determine if they differ regarding impairment of γTuRC recruitment.

Overall, I propose that interactors other than CDK5RAP2 make use of the GCP2 N-terminal region and these interactions contribute to γTuRC recruitment to the centrosome and stimulation of microtubule nucleation in interphase, possibly in a ch-TOG dependent manner.

ch-TOG activity in nucleation from CM1-γTuRC complexes

In addition to the regular MTOCs, we also observe an involvement of ch-TOG in ectopic microtubule nucleation from CM1-γTuRC complexes in the cytoplasm. While the ch-TOG depleted cells did not show a complete loss of cytoplasmic nucleation as seen in the case of GCP2 mutant cells in **Figure R3.3**, there was a considerable reduction in the number of microtubules formed. This result therefore suggests that the CM1 domain alone may not be sufficient for maximal nucleation activity from γTuRC and that ch-TOG adds to it. Given the conflicting results regarding the CM1-mediated nucleation activation from γTuRC *in vitro* (Choi et al., 2010; P. Liu et al., 2020; Rale et al., 2022; Thawani et al., 2020), it is important to explore further the contribution of ch-TOG/XMAP215 in such scenarios.

Notably, cooperation of a CM1-containing protein and a TOG domain protein in nucleation activation has also been observed in yeast: oligomerization of budding yeast γTuSC into an active nucleator mediated by CM1-containing Spc72 is enhanced by interaction with the ch-TOG orthologue Stu2 (Gunzelmann et al., 2018).

Together with the observation from **Figure R3.10**, where the ch-TOG dynamics were dependent on the same region in γTuRC as CM1 binding, it is possible that γTuRC activation by ch-TOG and CM1 binding may be mechanistically linked. While the presence of excess of CM1 in the cytoplasm does promote some level of ectopic microtubule nucleation, the presence of ch-TOG further enhances this process.

The observation that a majority of the cytoplasmic microtubules formed in ch-TOG depleted seem shorter compared to the control may suggest slower microtubule elongation. Therefore, it cannot be ignored that a reduced microtubule count in the depleted cells may also be due to microtubules that are too short to be detected, which could be interpreted as a polymerization defect rather than a nucleation defect. It has been reported that a truncated XMAP215 lacking only the C-terminus and retaining all five TOG domains did not support nucleation but was able to promote microtubule elongation similar to the full length protein (Thawani et al., 2018). Therefore, it can be tested in our system whether such a separation-of-function mutant of ch-TOG is able to support maximal activation of ectopic nucleation by γTuRC in cells overexpressing CM1. If not, this would indicate a specific role of ch-TOG in nucleation activation rather than merely a role in microtubule elongation.

Another point to consider is whether the mitotic defects associated with ch-TOG depletion would cause any stressful situation in the cells that could affect interphase nucleation activity assays. Future experiments involving specific removal of ch-TOG in interphase cells, for example by using an inducible degron-based system as discussed previously, would be ideal to avoid such possibilities.

In summary, our results regarding the crucial role of ch-TOG in microtubule nucleation at the centrosome and Golgi, as well as the *in vitro* evidences from other groups as discussed above, strongly support a role of ch-TOG/XMAP215 in γTuRC mediated microtubule nucleation, but a more detailed analysis of separation-of-function mutants would be desirable to distinguish between microtubule polymerase and nucleation activator activities.

A convergence of microtubule nucleation mechanisms

To address how γTuRC gets recruited to the MTOCs and how microtubule nucleation is stimulated from these complexes, I attempted to analyze different factors implicated in these processes, seemingly by very different mechanisms. Building on the results of a previous student in the lab that identified minimal regions within NINL to promote γTuRC recruitment vs microtubule nucleation, I was able identify ch-TOG as an interactor whose

recruitment correlated with the activity of NINL-dependent microtubule nucleation sites. This implicated ch-TOG in NINL-dependent nucleation.

In parallel we tried to test if the reported ability of ch-TOG and its orthologues to promote nucleation from γTuRC is also observable at centrosomal and non-centrosomal MTOCs in human cells. Apart from identifying a crucial role of ch-TOG in promoting microtubule nucleation at the interphase centrosome and Golgi.

Finally, probing the role of CM1-mediated interaction between γTuRC and CDK5RAP2 in nucleation at the centrosome revealed that more interactors likely make use of the CM1 binding site in γTuRC. Uunlike CDK5RAP2, these additional interactors are necessary for γTuRC recruitment to the interphase PCM as well as for efficient microtubule nucleation at this site. Again, I found an involvement of ch-TOG also in this scenario, likely via interaction with the N-terminus of GCP2, the same region that also binds CM1. The observation that ch-TOG enhances ectopic nucleation resulting CM1 overexpression further suggested that ch-TOG interaction via this CM1 binding region of γTuRC may be more broadly involved in microtubule nucleation from γTuRCs.

Overall these results converge towards a central role of ch-TOG in microtubule nucleation from γTuRC.

Perspectives

γ-Tubulin complexes are composed of a highly conserved group of proteins that regulate the assembly and organization of microtubules in eukaryotic cells. Yet there exists an astonishing diversity in the ways in which cells are able to control and manipulate the activity of these complexes. The regulation of assembly and activity of γ-tubulin complexes enable cells to generate and maintain specific organizations of microtubule networks.

The broad question that I tried to address in this thesis was to what extent diverse factors and mechanisms contribute to γTuRC recruitment and and activation at MTOCs. Among these, the roles of NINL, ch-TOG and CM1 domain-containing proteins were initially

considered. Based on our analyses, a central role of ch-TOG in promoting microtubule nucleation from γTuRC emerges as a common theme. However, our findings also open up several new questions that were left unaddressed due to limitations in time.

An important future goal would be to further validate our model that ch-TOG transient association via GCP2 N-terminus is required for ch-TOG to stimulate nucleation. For nucleation at the centrosome, this would also include identifying the adapter(s) at the PCM that enable stable binding of activated γTuRC, with CEP192 being a prime candidate. In addition, it needs to be addressed what the specific functions of SDA-associated and PCM-associated localizations of ch-TOG are.

While there are many unanswered questions arising from my observations, my results suggest a need to further dissect the interplay between ch-TOG and γTuRC to have a comprehensive understanding of microtubule nucleation in cells. In particular, given our observations from centrosomes, Golgi and artificial, ectopic MTOCs, it would be exciting to consider how ch-TOG might play a role in the diverse MTOCs seen in differentiated cells.

In summary, the results described in this thesis propose a crucial role for ch-TOG in γTuRC recruitment to the interphase centrosome as well as in activation of nucleation by γTuRC. I found this to be important for nucleation at centrosomal and non-centrosomal sites and to involve a transient association between ch-TOG and γTuRC, possibly mediated via the N-terminus of the GCP2 subunit of γTuRC.

Studying how different MTOC-associated proteins interact with and influence each other, and how these interactions vary between interphase, mitosis and during differentiation is crucial for a comprehensive understanding of how a dynamic microtubule organization is achieved in cells. Microtubule reorganization is central to cell differentiation and development. As such, proteins that are associated with microtubule nucleation, anchoring and organization have been associated with several developmental defects, one example being microcephaly present in disorders resulting from mutations in NIN or CDK5RAP2. Overexpression of microtubule- or centrosome-associated proteins including γ-tubulin, NINL and ch-TOG have been identified in several cancers. While dissecting how diverse

microtubule configurations are organized is highly relevant to development and disease, the sheer beauty of microtubule arrays observed in different cell types and the way by which a multitude of factors come together to assemble these makes studying these processes ever more interesting.

CONCLUSIONS

- NINL localizes to the subdistal appendages
- NINL accumulates and presents elongated distribution around duplicating centrioles
- NINL localizes to the centrosome independent of microtubules
- NINL may not be essential for centrosomal nucleation
- NINL N-terminus has the ability to recruit ch-TOG and MAP7D3
- ch-TOG is essential for γTuRC attachment to the PCM and microtubule nucleation in interphase
- ch-TOG associates with γTuRC transiently
- ch-TOG promotes microtubule nucleation from Golgi and ectopic CM1-γTuRC complexes
- CM1 domain from CDK5RAP2 is dispensable for γTuRC recruitment to both interphase and mitotic PCM
- CM1 domain from CDK5RAP2 is dispensable for centrosomal microtubule nucleation in interphase
- CM1 mediated activation of γTuRC is not the major pathway for nucleation at the centrosome in mitosis
- γTuRC tethering to interphase PCM is mediated via GCP2 N-terminus region
- GCP2 N-terminus promotes ch-TOG transient localization to the PCM
- GCP2 N-terminus mediates anchoring of microtubules at the interphase centrosome

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ANNEX

frontiers in Cell and Developmental Biology

MINI REVIEW
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Microtubule Anchoring: Attaching Dynamic Polymers to Cellular Structures

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Microtubules are dynamic, filamentous polymers composed of α- and β-tubulin. Arrays of microtubules that have a specific polarity and distribution mediate essential processes such as intracellular transport and mitotic chromosome segregation. Microtubule arrays are generated with the help of microtubule organizing centers (MTOC). MTOCs typically combine two principal activities, the *de novo* formation of microtubules, termed nucleation, and the immobilization of one of the two ends of microtubules, termed anchoring. Nucleation is mediated by the γ-tubulin ring complex (γTuRC), which, in cooperation with its recruitment and activation factors, provides a template for α- and β-tubulin assembly, facilitating formation of microtubule polymer. In contrast, the molecules and mechanisms that anchor newly formed microtubules at MTOCs are less well characterized. Here we discuss the mechanistic challenges underlying microtubule anchoring, how this is linked with the molecular activities of known and proposed anchoring factors, and what consequences defective microtubule anchoring has at the cellular and organismal level.

Keywords: MTOC, anchoring, microtubule, centrosome, nucleation

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INTRODUCTION

Microtubules, elongated, cylindrical polymers assembled from heterodimers of α- and β-tubulin, are major elements of the cytoskeleton that mediate a wide range of functions in cycling as well as post-mitotic, differentiated cells. The orientation of tubulin dimers within the microtubule lattice provides microtubules with an intrinsic polarity, exposing β-tubulin at the "plus-end" and α-tubulin at the "minus-end" (Alushin et al., 2014). Microtubule polarity is recognized by motor proteins to allow directed transport. Whereas most kinesins are plus-end-directed, the dynein motor and a few kinesins move towards the minus-end. Other proteins interact specifically with either of the two ends to modulate its dynamic behaviour through stabilization or destabilization (Akhmanova and Steinmetz, 2015). To function efficiently and to fulfil the specific needs of different cell types and cell cycle stages, microtubules are arranged into various types of arrays. These arrays differ in shape and distribution and may contain microtubules of uniform or mixed polarity and of variable dynamicity (Sallee and Feldman, 2021). To generate different types of microtubule arrays, cells employ microtubule organizing centers (MTOCs) (Sanchez and Feldman, 2017; Wu and Akhmanova, 2017; Paz and Lüders, 2018). MTOCs can be assembled at the cytoplasmic surfaces of various organelles. The best-known example is the centrosomal MTOC, which is assembled around centrioles, but other, typically membrane-bound organelles such as the Golgi or the nuclear envelope, can also acquire MTOC activity.

TABLE 1 | Major anchoring factors. Factors reported to be involved in anchoring microtubules at different MTCOs. Factors that likely affect anchoring more indirectly were not included. For each anchoring factor, identified by both the common name and organism-specific name, we indicate the ability to directly bind microtubules, the proposed role in anchoring, and the domains/regions involved in these functions. Question marks indicate cases where experimental data is not available. Abbreviations used: Sc–*S. cerevisiae*, Sp–*S. pompe*, At–*A. thaliana*, Hs–*H. sapiens*, Dm–*D. melanogaster*, Ce–*C. elegans*, SDA–Subdistal appendages, CC–Coiled coil.

Protein	Organism	Anchoring site	Microtubule binding	Role in anchoring	References
Stu2	Sc	Spindle pole body (SPB)	Direct binding (TOG domains; C-terminal region)	Minus-end stabilization; SPB and γ-tubulin complex binding (C-term. region)	Usui et al. (2003); Al-Bassam et al. (2006)
Spc72	Sc	SPB	?	Anchoring γ-tubulin complex and Stu2 (N-term. region)	Usui et al. (2003)
Pkl1	Sp	SPB (through Msd1)	Direct binding (motor domain)	Anchoring γ-tubulin complex	Yukawa et al. (2015)
Wdr8	Sp	SPB (through Msd1)	?	Anchoring γ-tubulin complex	Yukawa et al. (2015)
	At	Cortical microtubule array branch points	?	Minus-end stabilization at branch site	Yagi et al. (2021)
	Hs	Centrosome	?	Forms anchoring complex with Msd1; astral microtubule organization; spindle positioning	Hori et al. (2015)
Msd1	Sp	SPB	through Pkl1	Anchoring γ-tubulin complex	Yukawa et al. (2015)
	At	Cortical microtubule array branch points	?	Minus-end stabilization at branch site	Yagi et al. (2021)
	Hs (SSX2IP)	Centrosome	?	Anchoring γTuRC to PCM	Hori et al. (2014), Hori et al. (2015)
NEDD1	Hs	Centrosome	?	Anchoring γTuRC; centrosome binding (WD40 repeats); γTuRC binding (C-term. region)	Haren et al. (2006), Lüders et al. (2006), Manning et al. (2010), Muroyama et al. (2016)
FSD1	Hs	Centrosome (centriole central region)	Direct binding (SPRY domain)	Anchoring minus-ends (CC region for localization)	Tu et al. (2018)
Dynein complex	Hs	Centrosome; apical membrane	Direct binding (motor domain; CAP-Gly domain of p150^{glued} subunit)	Connecting microtubules to anchoring adapters	Quintyne et al. (1999), Askham et al. (2002), Culver-Hanlon et al. (2006), Kodani et al. (2013), Goldspink et al. (2017)
Ninein	Hs (NIN) Dm (Bsg25D)	Centrosome (SDAs, proximal end); apical membrane; nuclear envelope	?	Anchoring γTuRC (N-term. region); dynein adapter (multiple CC regions)	Mogensen et al. (2000), Delgehyr et al. (2005), Moss et al. (2007), Kodani et al. (2013), Goldspink et al. (2017), Rosen et al. (2019)
	Ce (NOCA-1)	Apical surface	?		Wang et al. (2015)
CAMSAPs	Hs Dm (Patronin) Ce (PTRN-1)	Centrosome; apical membrane; Golgi	Minus-end specific binding (CKK domain)	Minus-end stabilisation; interaction with other anchoring factors (C-term. CC region for localization)	Goodwin and Vale (2010) Jiang et al. (2014), Nashchekin et al. (2016), Toya et al. (2016), Wu et al. (2016), Yang et al. (2017)
NDEL1	Hs	Centrosome	?	Dynein regulator (C-terminal region)	Guo et al. (2006)
EB1, EB3	Hs	Centrosome; Golgi	Direct end binding (CH domain)	Connecting MTs to anchoring adapters and dynactin complex	Askham et al. (2002), Louie et al. (2004), Yan et al. (2006), Yang et al. (2017)
CAP350, FOP	Hs	Centrosome	?	Possibly docking EB1 at the centrosome, localisation of FSD1	Yan et al. (2006)
AKAP9 (AKAP450)	Hs	Centrosome; Golgi	?	Scaffold for MTOC assembly	Wu et al. (2016)
Myomegalin (MMG)	Hs	Golgi	?	Anchoring CAMSAP bound to minus-ends (N-terminal region)	Wu et al. (2016)
Spectraplakin	Hs (ACF7) Dm (Shot)	Apical membrane	Direct binding (GAR domain)	Localization and anchoring of CAMSAP3-bound minus-ends; CAMSAP3 binding (spectrin repeat region); actin binding (CH domains)	Leung et al. (1999), Wu et al. (2008), Nashchekin et al. (2016), Noordstra et al. (2016)
CLIP170	Hs	Apical membrane	Direct binding (CAP-Gly domains)	Ninein deployment	Folker et al. (2005), Ligon et al. (2006), Goldspink et al. (2017)
IQGAP1	Hs	Apical membrane	?	Ninein deployment	Goldspink et al. (2017)
RAC1	Hs	Apical membrane	?	Ninein deployment	Goldspink et al. (2017)
Piopio	Dm	Apical membrane	?	MTOC assembly	Brodu et al. (2010)

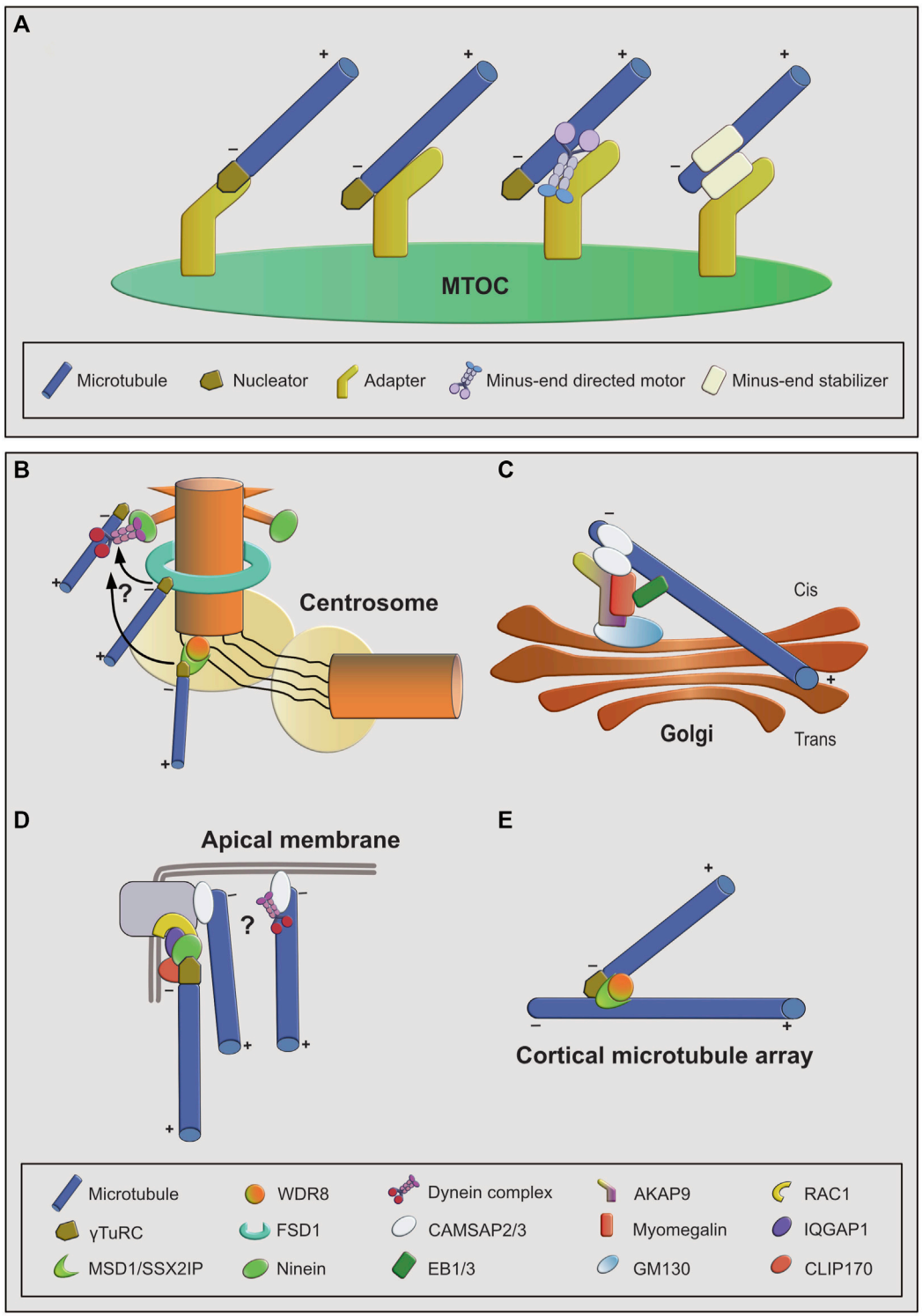

FIGURE 1 | Overview of microtubule anchoring sites and mechanisms. **(A)**: Conceptual overview of mechanisms by which microtubules can be anchored at MTOCs. From left to right: first, the nucleator may be part of the anchoring complex as a stabilizing minus-end cap. Anchoring to the MTOC may be achieved through an MTOC-bound adapter that interacts with the minus-end cap or with the microtubule lattice. Lattice interaction could be direct or indirect via a minus-end directed motor. Second, the nucleator may not be part of the anchoring complex. In this case anchoring is facilitated by an adapter protein interacting with a minus-end-bound, stabilizing protein. **(B–E)**: Examples of MTOCs and associated anchoring factors. **(B)**: At the interphase centrosome anchoring to the mother centriole is achieved through multiple mechanisms, involving ninein-dynein at the subdistal appendages, FSD1 in the central region, and MSD1-WDR8 in the proximal/PCM region. FSD1-and

(*Continued*)

FIGURE 1 | MSD1-mediated anchoring may be transient and minus-ends may be transferred to subdistal appendages. **(C)**: Anchoring at cis-Golgi membranes involves the AKAP9-myomegalin complex as adapter and CAMSAP2 as stabilizer at the microtubule minus-end that connects it to the adapter complex. EB proteins provide an additional way of connecting microtubules to the adapter complex through myomegalin. **(D):** At apical junction complexes and membranes in epithelial cells both nineinand CAMSAP-mediated anchoring mechanisms may act in parallel. **(E):** At branch points on plant cortical microtubules, MSD1-WDR8 complexes stabilize and anchor the minus-ends of newly nucleated microtubule branches to the lattice of the pre-existing microtubules.

A key component of most MTOCs is the microtubule nucleator γTuRC, which allows generation of new microtubules. Mimicking the structure of a microtubule in cross-section, the circular, helical arrangement of γ-tubulins in the γTuRC provides docking sites for tubulin heterodimers, promoting their lateral interactions and facilitating polymer formation (Moritz et al., 2000; Consolati et al., 2020; Liu et al., 2020; Thawani et al., 2020; Wieczorek et al., 2020; Zimmermann et al., 2020). Since soluble native and recombinant γTuRCs are intrinsically asymmetric and do not perfectly match the microtubule symmetry, they likely require activation, for example through a conformational change (Kollman et al., 2015). Alternatively, nucleation may be stimulated by cofactors that interact with and stabilize tubulin assembly intermediates on γTuRC such as members of the XMAP215 family of tubulin polymerases (Wieczorek et al., 2015; Flor-Parra et al., 2018; Gunzelmann et al., 2018; Thawani et al., 2018).

Apart from nucleation, the second, possibly most important activity of MTOCs is their ability to anchor microtubules. While most MTOCs also nucleate microtubules, MTOC activity could, in principle, also be carried out without nucleation, by capturing and anchoring of microtubules that were nucleated elsewhere. Indeed, it is the anchoring of microtubules at MTOCs that ultimately confers specific polarity and shape to microtubule arrays (Sanchez and Feldman, 2017; Wu and Akhmanova, 2017; Paz and Lüders, 2018). Several anchoring factors have been identified (**Table 1**), but in most cases their molecular functions are unclear and a mechanistic picture is still missing.

Here, we focus on the anchoring of microtubules at MTOCs and discuss our current knowledge regarding the molecules and mechanisms involved in this process. For an in-depth discussion of MTOCs and associated microtubule nucleation, we refer the reader to several recent reviews (Lee and Liu, 2019; Valenzuela et al., 2020; Wilkes and Moore, 2020; Lüders, 2021; Sallee and Feldman, 2021). We begin, by outlining conceptually how anchoring of microtubules may be achieved, and then, using various types of MTOCs and anchoring factors in different organisms as examples, discuss how available evidence supports these concepts. Finally, we highlight how defective microtubule anchoring impairs the microtubule cytoskeleton and may impair organismal development.

MOLECULAR REQUIREMENTS FOR MICROTUBULE ANCHORING

There are two basic requirements to be fulfilled by microtubule anchoring factors (**Figure 1A**). The anchoring proteins or protein complexes have to bind microtubules and, at the same time, interact with an MTOC. This linkage needs to be not only robust but also flexible, to resist the variable mechanical forces that act on microtubules as they extend away from the MTOC and serve as tracks for motor proteins. Importantly, to allow arrangement of microtubules with a specific orientation, binding of the anchoring factor to the microtubule needs to occur specifically at only one of the two ends. An additional challenge is the dynamic nature of microtubules. In microtubules assembled from pure tubulin *in vitro*, both minus- and plus-ends are dynamic and can undergo phases of growth and shrinkage. Thus, anchoring of microtubules likely involves stabilization and inhibition of microtubule end dynamics (Hendershott and Vale, 2014; Jiang et al., 2014; Akhmanova and Steinmetz, 2015). In the case of microtubules that are nucleated by γTuRC, the nucleator may form a stabilizing cap at their minus-end (Wiese and Zheng, 2000), leaving only the plus-end free to grow or shrink. This is consistent with the observation that anchoring of microtubules to MTOCs typically occurs via their minus-ends, whereas the plus-ends extend away from it and are more dynamic. Assuming that γTuRC remains bound to the minus-ends of newly nucleated microtubules, the nucleator itself could also provide anchoring function, as observed in reconstitution assays *in vitro* (Consolati et al., 2020). However, it seems that in cells anchoring usually involves additional factors (**Figures 1B–E**).

MICROTUBULE ANCHORING FACTORS AND MECHANISMS

Anchoring in Cycling Cells With a Central MTOC

A relatively simple microtubule network is found in budding yeast, where microtubules are organized by the spindle pole body (SPB), a single MTOC that is equivalent to the centrosome. Here anchoring of cytoplasmic microtubules was shown to involve ternary complexes composed of the SPB-bound adapter Spc72, the γ-tubulin-containing nucleation complex, and Stu2, a member of the XMAP215 family that also functions as nucleation stimulator (Usui et al., 2003; Gunzelmann et al., 2018). Following nucleation at the SPB, minus-ends are capped by γ-tubulin complexes and Stu2 may simultaneously interact with the nucleation complex and with the proximal wall of the newly nucleated microtubule (Usui et al., 2003). Similarly, in fission yeast γ-tubulin complexes cooperate with the XMAP215 homolog Alp14 to nucleate microtubules from the SPB and from the nuclear envelope, a second interphase MTOC, but a role of Alp14 in anchoring has not been described (Flor-Parra et al., 2018; Liu et al., 2019). Specifically during mitosis minus-ends of mitotic spindle microtubules are anchored through the coiled-coil protein Msd1 (Toya et al., 2007). Msd1 functions as part of a ternary complex with two other proteins, Wdr8 and the minus end-directed kinesin-14 motor Pkl1 (Yukawa et al., 2015). Minus-end-directed motor activity of

Pkl1 is used to transport Msd1 and Wdr8 towards the SPB, where the ternary complex interacts with γ-tubulin complexes and promotes minus-end anchoring. Interestingly, Pkl1, artificially tethered to the SPB, provided partial anchoring function, even in the absence of Msd1-Wdr8 and using a motor-defective rigor mutant of Pkl1 (Yukawa et al., 2015). One could speculate that in this scenario mutant Pkl1 may still be able to provide the two basic functions of anchoring factors outline above: localization to the SPB and interaction with microtubules. The role of Msd1 in anchoring is conserved. Human MSD1, also known as SSX2IP, was shown to interact with the nucleator γTuRC and promote microtubule anchoring at the centrosome, both in interphase and mitosis (Bärenz et al., 2013; Hori et al., 2014, 2015). Interestingly, at the centrosome MSD1 partially colocalizes with γTuRC, but does not colocalize with ninein/NIN, an established anchoring factor and component of subdistal appendages, structures specific to the mother centriole that have been implicated in microtubule anchoring. Moreover, a C-terminal MSD1 fragment that was sufficient to interact with γTuRC provides anchoring activity when artificially tethered to the pericentriolar material (PCM) in the proximal part of centrioles (Hori et al., 2014). This suggests that microtubules may be anchored not only at subdistal appendages and that anchoring could be coupled with nucleation. However, while human MSD1 also interacts with WDR8 (Hori et al., 2015), the molecular basis of its anchoring activity has not been revealed.

Anchoring of microtubules at the vertebrate centrosome involves subdistal appendages and the activity of the subdistal appendage protein ninein (Bouckson-Castaing et al., 1996; Mogensen et al., 2000; Dammermann and Merdes, 2002; Ou et al., 2002; Delgehyr et al., 2005; Lin et al., 2006). Several proteins described to affect anchoring may do so through altering subdistal appendage structure and/or ninein recruitment (Ibi et al., 2011; Kodani et al., 2013; Huang et al., 2017). Anchoring at subdistal appendages would imply that microtubules nucleated by γTuRC in the more proximally located PCM, which is considered to be the main nucleation site, would be transferred to the subdistal appendages for stable anchoring, possibly with γTuRC as a stabilizing minus-end cap (Delgehyr et al., 2005; Hori et al., 2014). However, γTuRC localizes not only to the PCM and there is some evidence that nucleation may also occur directly at subdistal appendages (Schweizer and Lüders, 2021). The finding that γTuRC interacts with ninein would be consistent with both models (Delgehyr et al., 2005). It should also be noted that centrosomal ninein is not restricted to subdistal appendages, but is also present at the proximal ends of both mother and daughter centrioles. The significance of this localization is not entirely clear but it may be related to ninein's role in centrosome cohesion (Mazo et al., 2016). How does ninein mediate microtubule anchoring? Ninein's N- and C-terminal regions were shown to mediate γTuRC-binding and centrosome targeting, respectively, but whether ninein can bind microtubules was not investigated (Delgehyr et al., 2005; Lin et al., 2006). However, ninein was shown to bind dynein (Casenghi et al., 2005) and, more recently, to function as dynein activator (Redwine et al., 2017). Dynein has been implicated in the centrosome targeting of several proteins, in some cases in the form of particles known as centriolar satellites (Kubo et al., 1999; Dammermann and Merdes, 2002; Prosser and Pelletier, 2020). In this case, however, dynein's ability to bind microtubules and move towards their minus-ends may be invoked by centrosome-bound ninein to anchor microtubules. Consistent with this possibility, several studies have linked dynein complexes with centrosomal microtubule anchoring (Quintyne et al., 1999; Guo et al., 2006; Kodani et al., 2013). Such a mechanism would have to ensure that dynein does not run off the microtubule once it has reached its minus-end. Indeed, at least *in vitro*, certain dynein complexes were observed to remain bound and accumulate at minus-ends (McKenney et al., 2014; Soundararajan and Bullock, 2014). Clearly, further work is needed to elucidate the potential cooperation between ninein and dynein in centrosomal minus-end anchoring.

Apart from MSD1 and ninein discussed above, a recent study has revealed another centrosomal protein, FSD1 (also known as MIR1 and GLFND), as microtubule anchoring factor (Tu et al., 2018). A comprehensive analysis showed that a coiled-coil domain at its N-terminus is sufficient for centrosome localization and that the B30.2/SPRY domain in the C-terminal part directly binds to and is required for anchoring of microtubules at the centrosome. Interestingly, FSD1 localizes in a circular fashion around centrioles, similar to subdistal appendage proteins, but positioned more proximally (Tu et al., 2018). Even though FSD1 localizes also around the daughter centriole, it promotes microtubule anchoring only at the mother centriole, pointing at the involvement of additional factors specific to the mother centriole. Notably, FSD1 and ninein are not dependent on each other for their specific localisations (Tu et al., 2018). The data suggest that FSD1, similar to MSD1, either extends the mother centriole-specific microtubule anchoring activity to the central portion of the cylinder or that it may be involved in the transfer of minus ends from proximally located nucleation sites to the subdistal appendage region for stable anchoring. Additional work is needed to clarify this issue.

Some anchoring factors share the ability to interact with γ-tubulin-containing nucleation complexes. This observation may indicate a mechanistic link between nucleation and anchoring and/or that γTuRC has two separate functions. Apart from providing a nucleation template, it may form a cap structure at minus-ends (Wiese and Zheng, 2000) that is used for microtubule anchoring. If so, distinct subpopulations of γTuRC may exist at centrosomes to mediate nucleation and anchoring, respectively. This was recently suggested to be the case in keratinocytes. In contrast to other cell types, where NEDD1 depletion robustly impairs centrosomal nucleation (Haren et al., 2006; Lüders et al., 2006), in keratinocytes nucleation activity is largely dependent on γTuRC in complex with the PCM protein CDK5RAP2, whereas γTuRC associated with NEDD1 is mainly used for anchoring (Muroyama et al., 2016).

Even in the presence of an active centrosome, MTOC activity associated with the Golgi may significantly contribute to microtubule network organization. This activity may be further enhanced when centrosome activity is compromised (Efimov et al., 2007; Miller et al., 2009; Rivero et al., 2009; Wu et al., 2016; Gavilan et al., 2018). CLASPs were initially proposed to provide anchoring

function to microtubules associated with the trans-Golgi network (Efimov et al., 2007), but more recent work suggested that they merely function in stabilization (Wu et al., 2016). AKAP9/AKAP450 is a central organizer of the MTOC at the cis-Golgi that recruits both nucleation and anchoring factors. γTuRC is used to nucleate Golgi-associated microtubules but does not seem to remain bound to their minus-ends. Instead, these are bound by the minus-end-stabilizing protein CAMSAP2, and tethered to Golgi membranes *via* myomegalin (Wu et al., 2016). Curiously, end-binding proteins EB1 and EB3, known as plus-end regulators, were shown to participate in tethering microtubules to Golgi membranes (Yang et al., 2017). Importantly, apart from Golgi-nucleated microtubules, CAMSAP2-decorated microtubules from other sites (e.g., nucleated and released from the centrosome) (Keating et al., 1997; Mogensen, 1999; Dong et al., 2017) can be captured and attached to the Golgi MTOC (Jiang et al., 2014; Wu et al., 2016). While the CAMSAP2-mediated minus-end binding mechanism is quite well understood (Atherton et al., 2017), the interplay with myomegalin and EBs for anchoring at the Golgi much less so.

Anchoring in Differentiated Cells With Distributed MTOCs

In metazoans, during cell differentiation the centrosome frequently loses its role as central microtubule organizer. As a result, in many specialized cell types microtubules are nucleated and anchored at more broadly distributed, non-centrosomal MTOCs (Paz and Lüders, 2018; Sallee and Feldman, 2021). An extreme case are plants, which lack centrioles altogether. In the plant interphase cortical microtubule array, for example, new microtubules are nucleated as branches from the lattice of pre-existing microtubules. Here the conserved Msd1-Wdr8 module was recently shown to anchor and stabilize microtubule minus-ends at the branch sites (Yagi et al., 2021). In addition, the Msd1-Wdr8 complex recruits katanin to the branch site, to allow severing and release of the newly nucleated microtubule branch. These activities are important for proper cortical microtubule array organization (Yagi et al., 2021).

Early work showed that during the differentiation of vertebrate polarized epithelia ninein expression is essential for cell polarization and formation of the apicobasal array of microtubules in these cells. As cells convert their centrosomal microtubule array to an apico-basal array, ninein is released from the centrosome to relocate anchoring function to an apical, non-centrosomal MTOC (Lechler and Fuchs, 2007; Moss et al., 2007; Bellett et al., 2009; Goldspink et al., 2017). In the epidermis this MTOC is formed in association with desmosomes at cell-cell junctions and is mediated by desmoplakin (Lechler and Fuchs, 2007). In columnar epithelial cells, ninein colocalizes with the adherens junction protein β-catenin. During differentiation, ninein associates with microtubules to be deployed at the apical MTOC in a process that depends on the plus-end interactor CLIP170 and cortical IQGAP1 and active Rac1 (Goldspink et al., 2017). Interestingly, once established, maintenance of the apico-basal microtubule array no longer required ninein. Experimental loss of ninein may be compensated for by apically localized CAMSAP2, and the dynactin subunit $p150^{Glued}$ (Goldspink et al., 2017), which has been implicated previously in anchoring at the centrosome (Quintyne et al., 1999; Kodani et al., 2013). Thus, different anchoring factors and mechanisms contribute and provide redundancy to apical anchoring of microtubules.

Apart from the apical membrane in polarized epithelial cells, ninein has also been identified at other non-centrosomal MTOCs, suggesting a broader role in microtubule anchoring. In mammalian multi-nucleated myotubes and in cardiomyocytes, ninein was identified as part of a non-centrosomal MTOC that forms during differentation at the nuclear envelope (Tassin et al., 1985; Bugnard et al., 2005; Srsen et al., 2009; Vergarajauregui et al., 2020; Becker et al., 2021). In muscle cells from *Drosophila* larvae ninein was also found in association with the perinuclear MTOC (Zheng et al., 2016). Later it was shown that in fly embryonic myotubes ninein cooperates with ensconsin/MAP7 in positioning nuclei along the myotube, which is important for muscle function (Rosen et al., 2019). More recently, a nuclear envelope-associated MTOC containing ninein was also described in *Drosophila* fat body cells, a cell type equivalent to liver adipocytes (Zheng et al., 2020). However, a formal demonstration that ninein mediates anchoring of microtubule minus-ends at these sites, is still lacking.

During neuronal differentiation ninein was observed to relocate from centrosomes to the cytoplasm in different neuronal compartments in the form of small granules, but no specific MTOC was identified (Baird et al., 2004; Ohama and Hayashi, 2009). Subsequently, ninein was revealed as a major transcriptional target of Sip1, a regulator of nervous system development. Loss of ninein phenocopied Sip1 deletion, and exogenous ninein expression was shown to rescue Sip1 deletion phenotypes, promoting axonal growth and branching by enhancing microtubule growth and stability (Srivatsa et al., 2015). It remains unclear though, whether these effects are related to a function of ninein in minus-end anchoring.

CAMSAP family members, which are not present in yeast and plants, can specifically recognize and stabilize minus-ends of non-centrosomal microtubules (Meng et al., 2008; Baines et al., 2009; Goodwin and Vale, 2010; Jiang et al., 2014; Atherton et al., 2017). Consistently, CAMSAPs are also associated with non-centrosomal MTOCs. In polarized epithelial cells in flies, worms and mammals, CAMSAP homologs were shown to contribute to the organization of apico-basal microtubule arrays that have their minus-ends anchored at non-centrosomal, apical MTOCs (Meng et al., 2008; Tanaka et al., 2012; Wang et al., 2015; Nashchekin et al., 2016; Ning et al., 2016; Noordstra et al., 2016; Toya et al., 2016). The contribution of CAMSAPs to apical minus-end anchoring may involve their ability to decorate microtubule minus-ends and to interact with spectraplakins that tether microtubules to the cortical actin network (Khanal et al., 2016; Nashchekin et al., 2016; Sanchez et al., 2021). In the larval epidermis in *C. elegans*, the CAMSAP homolog PTRN-1 functions redundantly with NOCA-1, a worm ninein homolog. Whereas NOCA-1 seems to work together with γ-tubulin, PTRN-1 likely stabilizes minus-ends in the absence of γ-tubulin (Wang et al., 2015). Similarly, in *Drosophila* fat body cells, ninein and patronin, the fly CAMSAP, function in parallel in organizing microtubule minus-ends at the nuclear envelope-associated MTOC. This function did not require γ-tubulin, even though it was also present at the nuclear envelope (Zheng et al., 2020). Recent

testing by induced degradation of a panel of candidate factors in *C. elegans* embryonic intestinal epithelial cells has confirmed significant redundancy in apical MTOC assembly and anchoring mechanisms (Sallee et al., 2018). A novel type of MTOC that lacked detectable γ-tubulin was recently described within varicosities of the basal process of highly polarized neural progenitors/radial glial cells in the brain (Coquand et al., 2021). CAMSAPs accumulated in the varicosities and knockdown of CAMSAP1/2 reduced microtubule growth from these sites and destabilized the entire basal process. Since the varicosities were positive for trans-Golgi and trans-Golgi-network markers, the microtubule-anchoring structures may be similar to those of the Golgi-associated MTOC (Wu et al., 2016; Coquand et al., 2021).

CONSEQUENCES OF MICROTUBULE ANCHORING DEFECTS

In cycling cells, centrosomal anchoring defects are expected to reduce the fidelity of mitotic spindle assembly, and impair the positioning of spindles, which relies on astral microtubule anchoring around centrosomes at the spindle poles. Anchoring defects at non-centrosomal MTOCs during differentiation, will likely interfere with proper microtubule network remodelling, which is required for the morphological and functional adaptations that cells undergo to carry out specific functions. Indeed, ninein depletion in cultured human cells prevents the organization of a radial, centrosome-centered interphase microtubule array, and causes multipolar spindles in mitosis (Dammermann and Merdes, 2002; Logarinho et al., 2012). In the early fly embryo, maternally provided ninein is required for proper mitotic spindle assembly, but it is not essential at later developmental stages (Kowanda et al., 2016; Zheng et al., 2016). Ninein in neural progenitors of the developing mammalian brain has a role in progenitor interkinetic nuclear migration, asymmetric centrosome inheritance, and progenitor maintenance (Wang et al., 2009; Shinohara et al., 2013). Depletion of the neural progenitor pool by mitotic defects has been shown to cause microcephaly in mouse models of Seckel syndrome, a developmental disorder that is caused by mutations in genes encoding centrosome proteins including ninein (Dauber et al., 2012; Marjanović et al., 2015). Additional work in ninein KO mice has revealed defects in the skin. Ninein loss was found to disrupt correctly oriented progenitor cell divisions and, during epidermal cell differentiation, the formation of non-centrosomal cortical microtubule arrays, impeding desmosome assembly and skin barrier formation. These defects are reminiscent of epidermis defects observed in *C. elegans* NOCA-1 (ninein) and PTRN-1 (CAMSAP) double loss-of-function mutants (Wang et al., 2015).

Several of the factors that contribute to anchoring microtubules at the interphase centrosome have also been implicated in the assembly of primary cilia, surface-exposed signalling organelles that form as an extension of the distal end of the mother centriole. Ciliary defects cause a group of developmental disorders known as ciliopathies. FSD1, ninein and KIF3A promote assembly of the ciliary transition zone, a critical step in ciliogenesis. At least in part this involves the formation and trafficking of centriolar satellites along mother centriole-anchored microtubules (Kubo et al., 1999; Kodani et al., 2013; Tu et al., 2018; Odabasi et al., 2019). Similar observations were made for MSD1, which is required for ciliogenesis in cultured cells and in zebrafish embryos (Hori et al., 2014). Subdistal appendage anchoring of microtubules is also important for proper positioning of cilia, which allows surface exposure of primary cilia (Mazo et al., 2016) and, in the case of motile cilia, coordination of ciliary beating (Kunimoto et al., 2012).

Loss of CAMSAP family members does not seem to affect centrosomes but rather non-centrosomal MTOCs. In flies, cortical patronin helps to define the anterior-posterior axis in the oocyte and, during abdominal epidermis formation, it is required for epithelial remodelling and proper abdomen development (Nashchekin et al., 2016; Panzade and Matis, 2021). Homozygous deletion of CAMSAP3's microtubule-binding domain in mice resulted in growth defects and, at the cellular level, in mispositioning of organelles. The architecture of polarized intestinal epithelial cells was only mildly affected, consistent with redundancy in apico-basal polarity organization (Toya et al., 2016). Analysis of CAMSAP loss-of-function in invertebrate and vertebrate models has revealed a wide range of phenotypes such as axon and dendrite growth and branching defects, reduced cell survival and organ size, or loss of ciliary motility (Chuang et al., 2014; Marcette et al., 2014; Richardson et al., 2014; Robinson et al., 2020; Liu et al., 2021; Yang and Choi, 2021). However, since CAMSAPs are likely general minus-end stabilizers rather than dedicated anchoring factors, some of these phenotypes may not necessarily result from anchoring defects, but, for example, from an overall reduction in microtubule density in CAMSAP-deficient cells (Jiang et al., 2014).

CONCLUSION AND OUTLOOK

The increasing interest in microtubule anchoring mechanisms has led to several important discoveries during recent years. This has also been facilitated by the use of invertebrate models such as *Drosophila melanogaster* and *C. elegans*, which are particularly useful for studying non-centrosomal MTOCs in the context of differentiated cells and tissues. The emerging picture is that MTOCs use multiple anchoring factors and mechanisms, often resulting in redundancy. While some mechanisms depend on the nucleator γTuRC, presumably as a stabilizing minus-end cap, others rely on γTuRC-independent anchoring, employing alternative minus-end stabilizers such as CAMSAP family members.

Important open questions are how microtubule minus-end binding is achieved, in particular for anchoring factors that do not directly bind to microtubules, and whether the presence of multiple anchoring mechanisms at a single MTOC simply provides redundancy or, alternatively, may indicate the presence of distinct anchoring sites that are specific for subsets of microtubules (Sallee et al., 2018). For example, dynamic microtubules may be anchored differently than more stable microtubules. This distinction may depend on the nucleation mechanism and site used to generate these microtubules, and may also involve specific post-translation modifications on their lattice (Janke and Magiera, 2020).

One major obstacle in studying minus-end organization at MTOCs is the crowded nature of these areas. Thus, when

addressing the above questions, the consequent use of super resolution techniques including expansion microscopy should enable researchers to probe anchoring sites with improved spatial resolution, to dissect single microtubule minus-ends, their post-translational modifications, and their associated molecules.

AUTHOR CONTRIBUTIONS

CV drafted the manuscript and prepared figures. JL conceived the review and prepared the final manuscript text.

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