The role of GCP8 in microtubule nucleation and cell cicle progression

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THE ROLE OF GCP8 IN MICROTUBULE NUCLEATION AND CELL CYCLE PROGRESSION

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Para mi familia y especialmente para Laura

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ABSTRACT

Microtubules (MTs) mediate a range of essential cellular processes including cell division, intracellular transport and organelle positioning, by forming specifically adapted arrays. Assembly, maintenance and remodelling of these arrays crucially depend on the de novo formation of MTs, termed nucleation, and its regulation in space and time. This process is mediated by the γ-tubulin ring complex (γTuRC), and impairment of the function of this complex is typically associated with mitotic defects. However, while the depletion of most γTuRC subunits compromises mitotic progression, the main role of GCP8/MZT2 seems to be the regulation of MT nucleation at the centrosome during interphase, although the molecular details are still unclear.

Therefore, the main goal of this project was to elucidate the mechanism by which GCP8 regulates interphase MT organization.

I found that GCP8 regulates MT nucleation by direct binding to a small segment at the N-terminal end of the GCP2 subunit of the γTuRC. Interestingly, using immunoprecipitation coupled to mass spectrometry, I have identified the MT depolymerase KIF2A as an interactor of GCP8. GCP8 recruits KIF2A to inhibit γTuRC-dependent nucleation at the centrosome and the Golgi apparatus. Moreover, I have observed that the depletion of GCP8 or KIF2A also results in loss of Golgi integrity. Compared to control cells, where the Golgi is typically organized as a ribbon on one side of the centrosome, Golgi membranes in knockdown cells appear more condensed and cluster tightly around the centrosome. Curiously, these defects promote cell cycle exit in non-transformed RPE-1 cells. Cell cycle exit is p53-independent and thus is unlikely to be caused by problems during mitotic progression. Strikingly, depletion of GCP8 or KIF2A impairs mTOR activation which may explain the cell cycle progression defect.

In summary, by analysing the contribution of GCP8 in the regulation of interphase MT nucleation, I have described an unexpected mechanism that links MT-dependent organization of the centrosome-Golgi axis to G1/S progression and involves regulation of mTOR.

RESUMEN

Los microtúbulos (MTs) regulan una amplia variedad de procesos celulares, incluyendo la división celular, el transporte intracelular y la posición de los orgánulos, al formar una red específicamente adaptada. El ensamblaje, mantenimiento y remodelación de estas redes depende de la formación de nuevos MTs, denominada nucleación, y de su regulación en el espacio y tiempo. Este proceso está mediado por el por el complejo en anillo de γ-tubulina (γTuRC) y defectos en la función de este complejo están directamente asociados con problemas mitóticos. Sin embargo, pese a que la depleción de la mayoría de las subunidades del γTuRC compromete la progresión de la mitosis, la función principal de GCP8/MZT2 parece ser la nucleación de MTs desde los centrosomas durante la interfase.

Por este motivo, el objetivo principal de mi tesis fue descifrar los mecanismos moleculares por los que GCP8 regula la organización de MTs durante la interfase.

GCP8 regula la nucleación de MTs mediante la unión a través del externo N-terminal de la subunidad GCP2. Mediante técnicas de inmunoprecipitación acoplada a espectrometría de masas, identifiqué la proteína KIF2A, una despolimerizadora de MTs, como un interactor de GCP8. KIF2A es reclutada por GCP8 para inhibir la nucleación dependiente del γTuRC en los centrosomas y en el aparato de Golgi. Además, observé que la depleción tanto de GCP8 como de KIF2A da lugar a la pérdida de la integridad del Golgi, el cual presenta, a diferencia de las células control, una condensación de sus membranas estrechamente agrupadas alrededor del centrosoma. Curiosamente, estos defectos dan lugar a la salida del ciclo celular en células RPE-1. Esta salida es independiente de la vía de señalización p53 por lo que es improbable que estos defectos se deban a problemas durante la progresión de la mitosis. Sorprendentemente, la depleción de GCP8 o KIF2A impide la activación de la mTOR lo que podría explicar los defectos en la progresión del ciclo celular.

En resumen, al analizar la contribución de GCP8 en la regulación de la nucleación de MTs durante la interfase he conseguido describir un inesperado mecanismo que vincula la organización de MT dependiente del eje centrosoma-Golgi con la progresión de las fases G1/S del ciclo celular, involucrando a la vía de señalización de mTOR.

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INTRODUCTION

1) MT structure and dynamics

MTs are, together with actin, septins and intermediate filaments one the four components of the eukaryotic cytoskeleton. In contrast to intermediate filaments, which are made of several fibrous proteins and are extremely stable, septin, actin and MTs are dynamic structures composed of a single type of globular proteins that undergo changes between phases of assembly and disassembly. All four cytoskeleton elements cooperate to provide mechanical support to the cell and are involved in many cellular processes such as cell division, molecule transport, organelle tethering and organization, migration and cell signaling.

MTs are hollow tube-shape structures with a diameter of around 25nm assembled from heterodimers of α- and β-tubulin (Linda A. Amos, 1974; Kollman JM, 2011)**.** The tubulin subunits associate longitudinally to form a protofilament, which additionally make lateral contacts to generate the typical circular, 13-protofilament symmetry observed in MT cross-sections. The head to tail assembly of α- and β-tubulin confers MTs an intrinsic polarity with β-tubulin exposed at the more dynamic plus end and α-tubulin facing the more stable minus end (Allen C & Borisy GG, 1984; Akhmanova A & Steinmetz MO, 2008)

α- and β-tubulins are closely related isotypes with GTP binding capacity at conserved sites. The GTP binding site of α-tubulin (N-site, non-exchangeable) localizes at the interspace between tubulin monomers within heterodimers and is not hydrolyzed. However, β-tubulin exposes its GTP binding site (E-site, exchangeable) on the dimer surface, where GTP is hydrolyzed after polymerization (Nogales E, 1998). This hydrolysis results in highly dynamic MTs that switch between growing phases (when β-tubulin is bound to GTP) and shrinking phases (when the GTP binding site of β- tubulin gets hydrolyzed), a phenomenon known as dynamic instability (Tim Mitchison & Marc Kirschner, 1984). The transition from growing to shrinkage is called catastrophe, while the transition from shrinkage to growing is called rescue. Additionally, among these phases, MTs can reach a pause state as well, where neither grow nor shrinkage takes place (Figure 1).

MTs can form spontaneously in vitro at a certain α-β-tubulin concentration. However, MT assembly in vivo is a tightly regulated process that requires a nucleator to control the

formation of new MTs in time and space (Lüders J & Stearns T., 2007; Teixidó-Travesa N, 2012; Tim Mitchison & Marc Kirschner, 1984).

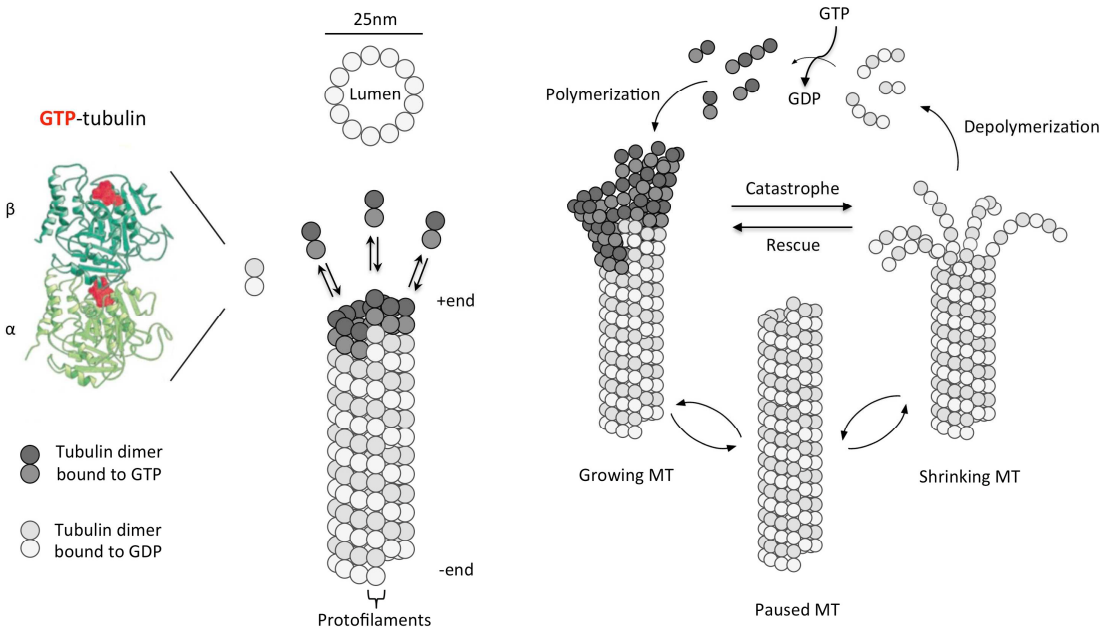


Figure 1. MT composition and dynamic instability. MTs are tubular structures assembled from α- and β-tubulin heterodimers in a GTP-dependent manner. During growing phases, GTP-tubulin dimers are incorporated at the plus end to subsequently be hydrolysed after polymerization. Therefore, the MT lattice is mainly composed of GDP-tubulin. By contrast, a catastrophe event is characterized by the release of tubulin dimers leading to the shrink of the MT. A third stage called pause takes place when MTs neither polymerize nor depolymerize. Inspired by (*Akhmanova & Steinmetz 2008*)

A proper control of MT dynamics is fundamental to accurately cover the huge variety of MT functions. To perform a specific function MT dynamics can be regulated by the binding of different proteins that can be classified in MT-associated proteins (MAPs) and motor proteins (Desai A, Mitchison TJ., 1997).

1.1) MAPs

MAPs can be classified according to their localization on MTs, where they can promote MT growth, depolymerization or stabilization. A superfamily of MAPs are the plus-end tracking proteins (+TIP), which includes proteins that promote MT growth by recruiting tubulin dimers at the plus end, such as XMAP215 (Brouhard GJ, 2008*;* Al-Bassam J, 2012) or by detecting the nucleotide state of tubulin and modulating the structure of the growing end, such as the well-characterized EB1 (Yulia Komarova, 2009*;* Zanic M, 2013; Maurer SP, 2014*;* Sebastian P. Maurer, 2012). Additionally, when localized at the plus end, cytoplasmic linker-associated proteins (CLASPs) have been shown to stimulate MT growth by inhibiting catastrophe (Al-Bassam J K. H., 2010*;* Takashi Moriwaki, Gohta Goshima, 2016*;* Yu N, 2016). However, CLASPs can also localize at the MT lattice and promote MT stabilization (Wittmann T1 & Waterman-Storer CM, 2005)(Figure 2).

Apart from stimulating MT growth, for proper regulation of MT dynamics catastrophe events must be also be stimulated under certain cellular conditions. Depolymerase activity has been described for some kinesin protein members, including kinesin-8, kinesin-14 or kinesin-13, the latter being the most extensively studied (Desai A, 1999*;* Gardner MK, 2011*;* Sproul LR, 2005*;* Akhmanova A & Steinmetz MO., 2015). An example is MCAK, which removes tubulin dimers using the hydrolysis of ATP (Hunter AW, 2003), or KIF2A, which I will explain in more detail below.

On the other hand, those MAP members that localize at the MT lattice commonly promote MT stabilization (as I commented before for CLASPs proteins). This is particularly important in neurons where the transport of the different cargos requires the stabilization of MT, e.g. in the dendrites by MAP2 and in the axon by TAU (Dehmelt L1 & Halpain S., 2005). Moreover, problems in the binding of some of these proteins, such as the hyper-phosphorylated form of TAU, have been linked to some neurodegenerative diseases like Alzheimer (Grundke-Iqbal I, 1986)

Finally, a third group of MAPs comprises the minus end localized proteins. This end has been typically related as the less dynamic MT pole. In fact, it is mainly characterized as the region where new MT formation, termed MT nucleation, takes place through its association

with the γ-tubulin ring complex (γ-TuRC), as I will described in more detail below. However, the minus end dynamics can be also controlled through the binding of CAMSAP protein family, which are well described as minus end stabilizing (Goodwin SS & Vale RD., 2010*;* Jiang K, 2014*;* Akhmanova A H. C., 2015), or by members of kinesin-13 family, which have been shown to stimulates MT depolymerization at this end as (Goodwin SS, Vale RD., 2010) (Figure 2).

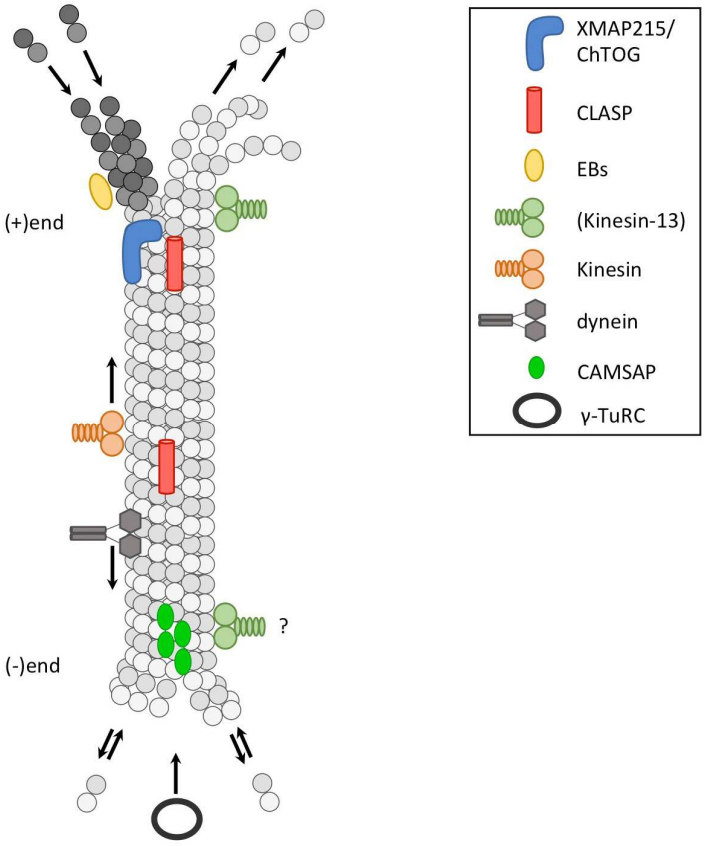


Figure 2. Functional classification of MAPs and motor proteins. In this schematic illustration MAPs are classified based on region where they localize and its function. Thus, plus tip tracking proteins include EBs, XMAP215 or CLASP (stimulate MT growth) and kinesin-13 family (promote MT catastrophe). At the MT lattice while CLASP stabilize the MTs, motor proteins are involved in cargo transport such as kinesin proteins (towards the plus end) and dynein (towards the minus end). Finally, minus end localizing MAPs include CAMSAP (minus end stabilization), the γTuRC (nucleation and stabilization) and kinesin-13 family (minus end depolymerization in vitro).

1.2) Motor Proteins

The MT motor proteins, although they can also regulate the MT dynamics as I commented before, they are mainly involved in the transport of cargos by moving along the MT lattice through the hydrolysis of ATP. This intracellular transport along MTs is particularly important for the cells to maintain their specific functions and morphologies. Therefore, MTs have been typically considered as the cellular highways essential for the control of the delivery, localization and function of different cargos such as, proteins, vesicles or organelles. We can separate motor proteins in two different groups. Those that move towards the plus end (anterograde), which includes most of the kinesin superfamily, and minus end (retrograde) directed transport proteins, being dynein the most important (Figure 2) (Paschal BM, 1987*;* Vale RD, 1985*;* Hirokawa N1 & Noda Y., 2008*;* RH, 2009). Thus, MT polarity regulates the directional movement of these proteins along the MT lattice. MT organization is very varied between cell types and its maintenance is crucial to control the delivery of cargos either during interphase and mitosis or in post-mitotic cells.

1.3) Post-translational modification (PTMs)

A further mechanism to regulate the specialization of MT function is based on the expression of different tubulin isotypes and post-translational modification, which can generate MTs with variable properties. In most organisms, α-tubulin and β-tubulin are encoded by multiple genes such in mammals, in which nine genes for each tubulin isotype have been described. However, little is known about the real contribution of the different tubulin isotypes in the specialization of MT functions (Gadadhar S, 2017).

On the other hand, PTM´s are thought to regulate MT behavior directly or indirectly by regulating the binding of other MAPs (Janke C & Bulinski JC., 2011). Many different tubulin modifications have been described, some of which are relatively well characterized such as acetylation, (poly)glutamylation, (poly)glycylation or detyrosination. Altogether, MT polymers can be modified in a heterogeneous manner leading to a high level of complexity with implications that are still poorly understood (Song Y & Brady ST, 2015). Moreover, given the range of disease associated to tubulin mutations or the different phenotypes

described in knockout organisms for tubulin-modifying and de-modifying enzymes demonstrate the importance of advancing our knowledge in this field (Figure 3).

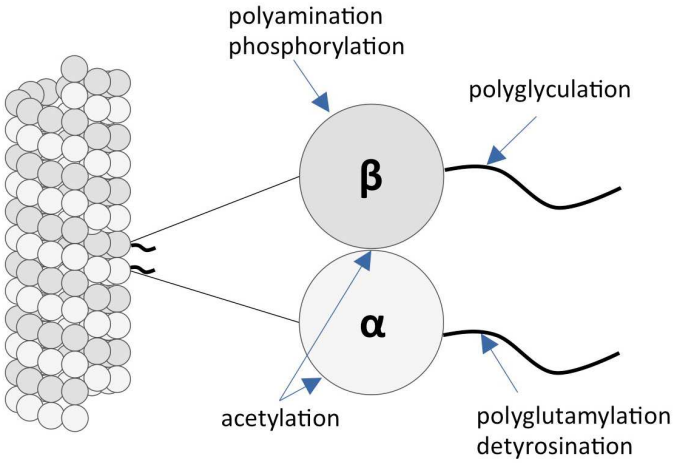


Figure 3. Tubulin PTMs. Schematic representation of the α-β-tubulin PTMs. While tubulin acetylation, phosphorylation and polyamination take place within tubulin dimers, polyglyculation, polyglutamylation and detyrosination occur at the C-terminal tails of α/β tubulin. Adapted from Janke, 2014.

2) MTOCs

In cells, MT nucleation is tightly regulated in space and time at specific cellular structures known as MT-organizing centers (MTOCs). This function is mainly dependent on the presence of γTuRCs at the different MTOCs, which I will classify as centrosomal and non-centrosomal MT organizing centers. Moreover, apart from its ability to concentrate MT nucleation events, an MTOC must be able to anchor and stabilize MTs. The number and the relevance of each specific MTOC varies between organisms, cells types and cell stages, but they all need to be properly regulated to ensure correct MT organization.

2.1) Centrosomal MTOC

More than a century ago, Theodor Boveri described the importance for cell division and fertilization in *Ascaris megalocephala* (now *Parasacaris equorum*) of a new organelle that he called "the centrosome" (Boveri, 1888). Over the years, this organelle has been considered the main MTOC in animal cells, having key roles during interphase and mitosis by regulating cell polarity and motility, spindle assembly, chromosome segregation and cytokinesis (M, 2012; Conduit PT, 2015)

The centrosome is composed of two barrel-shaped centrioles characterized by a conserved nine-fold symmetry and surrounded by an electron-dense matrix termed pericentriolar material (PCM). Both centrioles differ by the present of additional structures (appendages) in one of them, which is typically referred as the mother centriole (Figure 4). While the distal appendages play a role during cilia formation, the sub-distal appendages have been proposed to regulate MT anchoring (De Harven E & Bernhard W, 1956; Doxsey, 2001; Bornens, 2002)

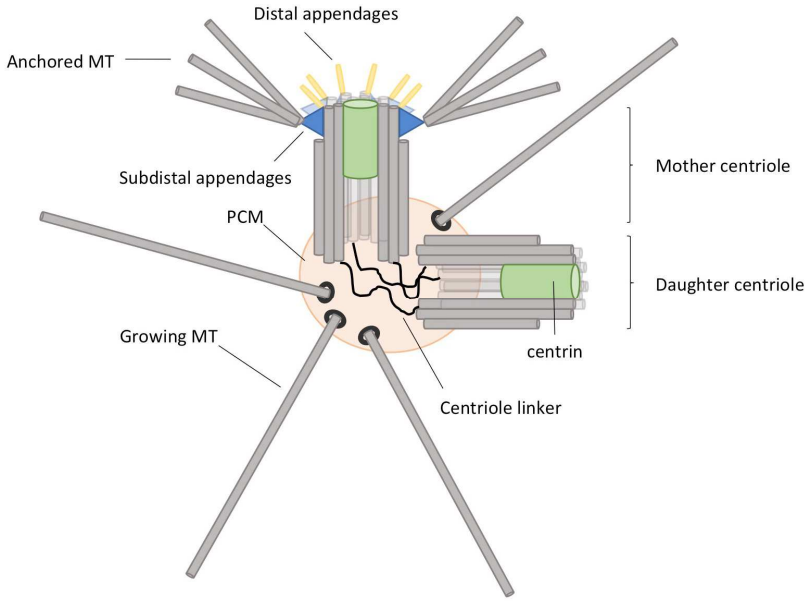


Figure 4. The centrosome structure. Both centrioles, the mother and the daughter, are made of 9 MT triplets arranged circumferentially and associated by interconnecting fibers. Into the distal ends localize centrin, a protein required for centrosome duplication. The mature centriole contains two additional structures composed of multiple proteins called appendages (distal and sub-distal). The PCM, a protein matrix containing γTuRCs, surrounds both centrioles and is the region associated to MT nucleation events.

Traditionally, the PCM was described as an amorphous structure composed of hundreds of different proteins. However, the use of new advanced microscopy techniques revealed that the PCM is in fact highly organized in several layers where γTuRC complexes and γTuRC regulators are concentrated. Therefore, the PCM has been broadly accepted as the centrosomal region where MT nucleation takes place (Lüders J, Stearns T., 2007*;* Woodruff JB, 2015)

Similar to DNA replication, the centrosome must duplicate only once per cell cycle, so that during mitosis each daughter cell receives one centrosome. During interphase, several changes, including duplication, maturation and separation, must occur on the centrosomes to ensure spindle bipolarity (Figure 5). Defects in this highly regulated process have been associated to several diseases including aneuploidy and cancer (Nigg E &, Holland AJ, 2018*;* Gogendeau D, 2015).

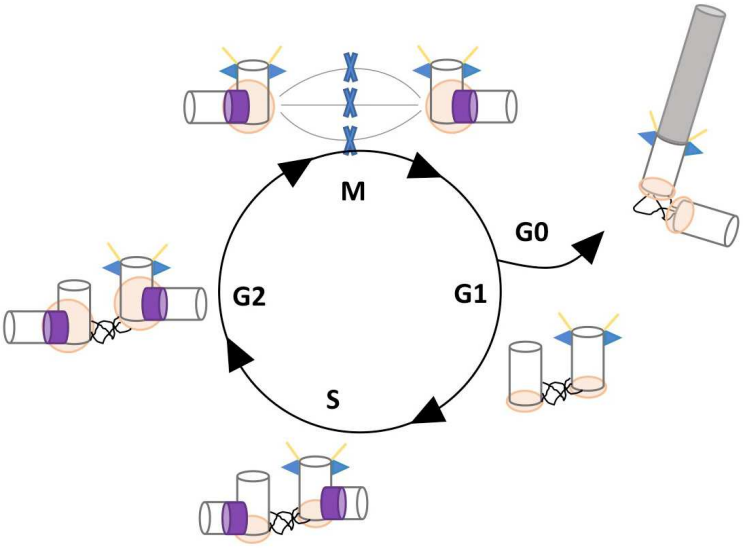


Figure 5. Centrosome cycle. The centrosome cycle is tightly linked to the cell cycle and comprises several steps, mainly regulated by phosphorylation. After mitosis, the centrioles disengage and a proteinaceous linker is established during G1. Then, throughout S-phase, centrosome duplication and elongation initiates with the formation of a procentriole from a cartwheel-like structure (represented in lila) on the wall of the pre-existing centrioles. Later, during G2, centrosome maturation starts with the accumulation of multiple PCM proteins. Finally, in late G2-phase the centrosome separate to form a bipolar spindle during mitosis. Once the cell stops proliferating (G0) the centrosome moves to the cellular surface to act as a basal body for primary cilia formation.

In other organism, such as fungi, the spindle pole body, which is a functional equivalent of the centrosome, is embedded in the nuclear envelope and is required for proper MT network formation (M., Jaspersen SL & Winey, 2004).On the other hand, plant cells lack a centrosome equivalent but they nevertheless are able to arrange highly organized MT arrays (Wasteneys GO & Ambrose JC., 2009).

In any case, the functional relevance of the centrosome, or equivalents, as a major MTOC relies on the presence of γ-tubulin, and the γ-TuRC, as a key component of the PCM essential not only for MT nucleation (Tim Stearns, 1991) but also for the anchoring and stabilization of MTs by capping the minus ends (Paz J & Luders L, 2018).

Beyond MT nucleation, MT organization at the centrosomes requires the stabilization and anchoring of the newly formed MTs. So far, two major candidates have been proposed to regulate the anchoring process. Experiments performed with recombinant NEDD1, a subunit of γTuRC, showed that fusing the C-terminal half of NEDD1 to the desmosome-targeting domain of Desmoplakin was sufficient for anchoring MT at the cellular cortex (Muroyama A, 2016). On the other hand, Ninein was proposed to stabilize and anchor the MT at the sub-distal appendages of the centrioles (Delgehyr N, 2005).

2.2) Non-centrosomal MTOC

For many years the centrosome was considered indispensable not only for the radial distribution of MTs during interphase but also for setting up the bipolar spindle during mitosis. However, even though centrosomal MT organization is required for the formation of astral MTs at the spindle poles and for spindle positioning (Betschinger J1 & Knoblich JA., 2004 Cowan CR & Hyman AA., 2004*)*, it has been shown that somatic cells can undergo mitosis without centrosomes (Khodjakov A, 2000). During mitosis, MT nucleation can take place at additional sites such as the chromatin (Witt PL, 1980*,* Heald R, 1996*)*, or from pre-existing MTs (Goshima G, 2008), suggesting that these non-centrosomal pathways can compensate for the lack of centrosomes and are sufficient for mitotic spindle assembly. In

fact, mutant flies can develop to adult stages without centrioles, although they finally die after birth because of lacking sensory ciliated neurons (Basto R, 2006)

Moreover, plant cells, which lack centrosomes, MTs are highly organized in a non-centrosomal manner. Instead these cells nucleate MTs at other cellular structures such as the nuclear envelope or the cellular cortex (Masoud K, 2013*;* Hamada, 2014). Interestingly, these two non-centrosomal sites are also used as MTOCs in some differentiated animal cells, in which the centrosome is relatively inactive. Muscle cells use the nuclear envelope and the Golgi as main MTOCs and epithelial cells organize MT at the apical membrane (Bugnard E1, 2005*;* Tassin AM, 1985*;* Gimpel P, 2017; Martin M & Akhmanova A, 2018)

Nevertheless, one of the best-characterized non-centrosomal MTOCs is the Golgi apparatus, which is particularly relevant for MT nucleation during interphase (Karine Chabin-Brion, 2001), although it also participates in mitotic spindle assembly by releasing TPX2 from the repressor factor importin α (Wei JH, 2015).

MT nucleation at this organelle is important for the higher-order organization of the Golgi into a ribbon (at least in vertebrates) and for its asymmetric and juxta-centrosomal positioning. This configuration is crucial for polarized trafficking to promote cellular polarity and directional migration (Hurtado L, 2011; Tatiana Vinogradova, 2009). As in the case of the centrosome, MT nucleation at this organelle relies on the γ-TuRC, whereas the regulation machinery is slightly different, as I will discuss in more detail below.

2.2.1) Golgi structure and function

The Golgi apparatus is membrane-bound organelle found in most eukaryotes mainly involved in the secretory pathway where proteins and lipids derived from the endoplasmic reticulum (ER) are post-transnationally modified at the Golgi and sorted to specific cellular locations (Figure 6). It is closely associated to the centrosomes and over the last years has been also well establish its role as a MTOC (Karine Chabin-Brion, 2001). Golgi-derived MTs are important not only to maintain the integrity of this organelle but also for cell polarity and migration (Wu J, 2016*;* Shicong Wang, 2014).

Regarding the Golgi structure, electron microscopy images show that it is composed of compact stacks of flat cisternae connected by tubular membranes (Pierfrancesco Marra, 2007). MTs are important for the ribbon organization (Figure 7) and correct positioning. Although they look morphologically similar, each cisterna contains a set of different enzymes that confer specific modification to the incoming substrates (BS, Papanikou E & Glick, 2014).

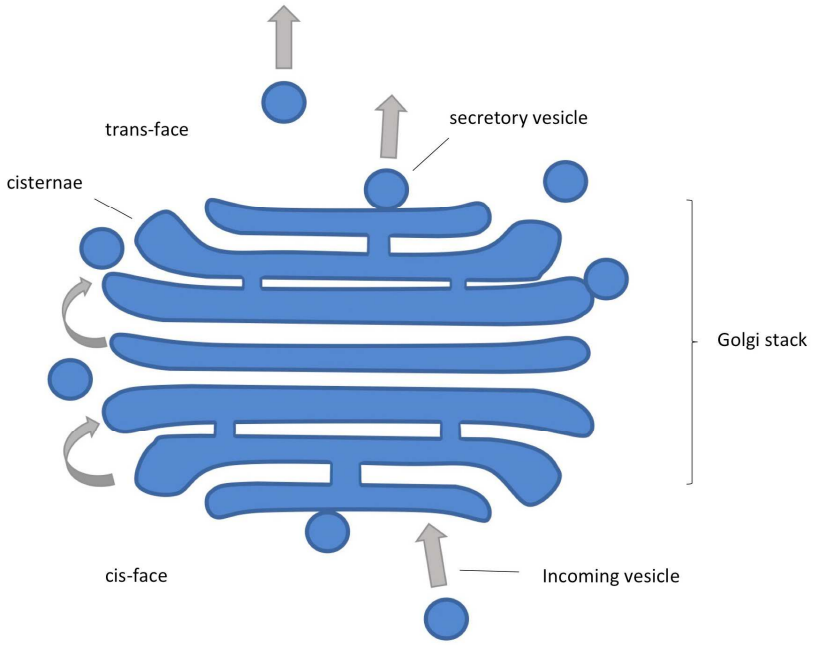


Figure 6. The Golgi apparatus. The Golgi stack is made of several flattened polarized cisternae membranes with a cis- and a trans-face representing the entry site from incoming cargos from the ER and the cargo exit site to specific cellular destinations respectively.

An interesting feature of the Golgi structure is it changes from individual stacks in protists, plants and invertebrates into a compacted Golgi ribbon in vertebrates (Mary J. Klute, 2011). Since the Golgi can function as dispersed stacks in many organisms, the significance of the ribbon organization is still unclear. One possibility is that the ribbon organization of the Golgi in vertebrates reflects a higher cellular complexity including more complex trafficking and glycosylation (Makhoul C, 2018). Indeed, changes in the Golgi architecture have been linked to several human diseases, including neurodegenerative (Vinod Sundaramoorthy, 2015).

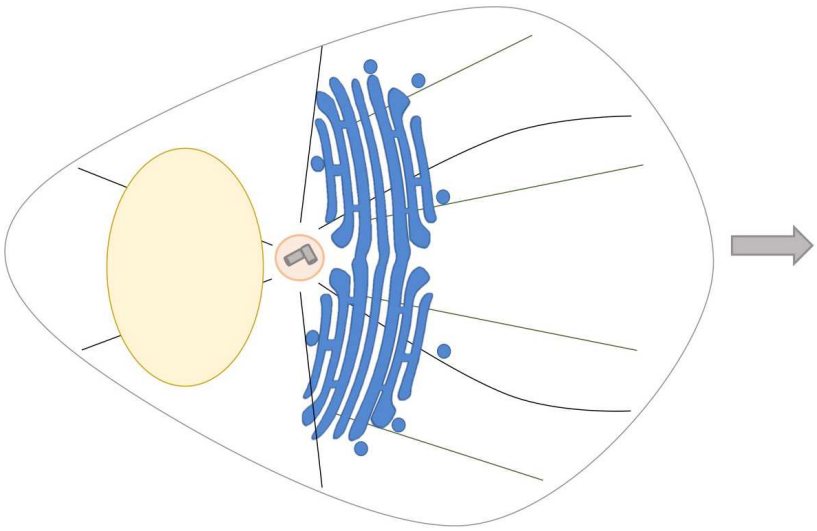


Figure 7. The Golgi ribbon structure and function. The Golgi apparatus is mainly involved in the post-translational modification of secretory and membrane proteins and sort them to the corresponding cellular destination. In vertebrates, this organelle adopts a higher-ordered structure called the Golgi ribbon, which is typically composed of compact stacks laterally interconnected and is localized next to the nucleus in a pericetriolar region of the cell. While the centrosome has been considered the main MTOC, MTs derived from the Golgi apparatus are essential to establish cell polarization and directional migration.

Surprisingly, around two hundred signaling genes have been implicated in regulating Golgi ribbon organization (Chia J, 2012; Farhan H, 2010) and, potentially, some of the signaling pathways implicated may in turn also be regulated by this organelle. For example, it has been recently proposed that problems in the ribbon organization may negatively regulate the mechanistic target of rapamycin (mTOR) pathway (Gosavi P, 2018).

mTOR is a serine-threonine kinase with a pivotal role in a range of cellular processes including cellular growth, proliferation, survival, cell migration, protein translation and metabolic processes (Sabatini, 2006)mTOR exists in two major multiprotein complexes namely mTORC1 and mTORC2, which regulate different cellular functions (Sarbassov D, 2005; Mecca C, 2018).

Under certain stress conditions, such as nutrient deprivation, eukaryotic cells have developed protective mechanism to inhibit cellular growth in order to optimize the usage of limited energy resources. This mechanism involves autophagy, which is in charge of mobilizing intracellular nutrients supplies (Jung CH, 2010).One of the main cellular sensors

involved in the regulation of this response is mTOR, concretely the mTORC1 complex, which has been proposed to localize at both the lysosomal surface and the Golgi apparatus (Figure 8) (Hao F, 2018; Yasemin Sancak, 2011), and must be inhibited to stimulate autophagy (Scott RC, 2004). However, under proliferative conditions, mTORC1 is activated by the small GTPase protein Ras homolog enriched in brain (Rheb), stimulating protein synthesis and cell growth by phosphorylating the eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) and the ribosomal protein S6 kinase (S6K).

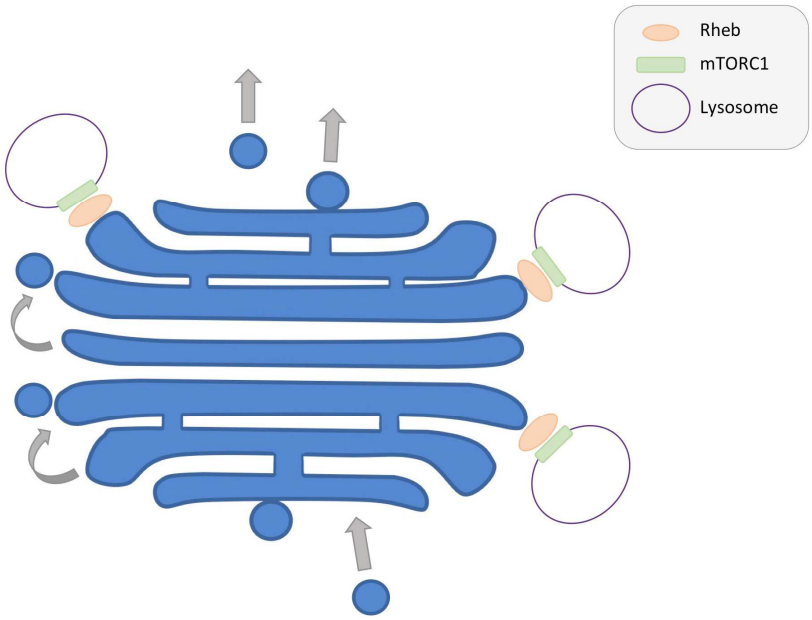


Figure 8. mTOR activation at the GLCS. Rheb partially localizes on the Golgi apparatus. The lysosome containing mTORC1 moves to the Golgi leading to the activation of mTORC1 by Rheb. Adapted from Hao F. et al., 2018.

Curiously, it has been recently shown that in some cases, mTOR inactivation stimulates primary cilia formation, also termed ciliogenesis, through the up-regulation of the cyclin-dependent kinase inhibitor p27 (Takahashi K, 2018). Moreover, due to its role in the regulation of vesicular trafficking, problems in the Golgi function have been associated to defects in cilia formation by impairing the delivery of ciliary proteins to the cilium (David Asante, 2013; John A. Follit, 2006)

Overall, considering the large variety of cellular processes where this organelle is implicated, a better understanding of Golgi homeostasis remains an important challenge for our comprehension of multiple human diseases.

3) Primary cilia

Cilia are hair-like structures that emanate from the apical surface of many different cell types and can be classified in motile cilia and primary cilia. Whereas motile cilia are involved in cellular locomotion and fluid flow over epithelia, primary cilia carry out sensory functions by receiving external signals from the environment, such as growth factor or hormones, to control intracellular signalling pathways and gene expression (Mitchison HM & Valente EM, 2017). Thus, primary cilia are directly involved in the regulation of a variety of cellular processes, including embryonic development or metabolism. Defects in cilia have been linked to different types of diseases collectively referred to as ciliopathies (Ishikawa H & Marshall WF., 2011; Yenniffer Ávalos, 2017)

The formation of primary cilia is tightly associated with the centrosome and the cell cycle (Figure 9). In cycling cells, centrosomes work as a MTOC during interphase and mitosis, while in differentiated cells the centrosome templates ciliogenesis. During this process the centrioles, acting as basal bodies, move towards the cellular surface, where the distal appendages on the mother centriole mediate docking to the plasma membrane (Figure 9). The role of the sub-distal appendages is less clear, although they have been related to primary cilia disassembly after cell cycle re-entry and to maintain the cilia in “submerged configuration” associated with the Golgi apparatus (Gregory Mazo, 2017*;* Tatsuo Miyamoto, 2015).

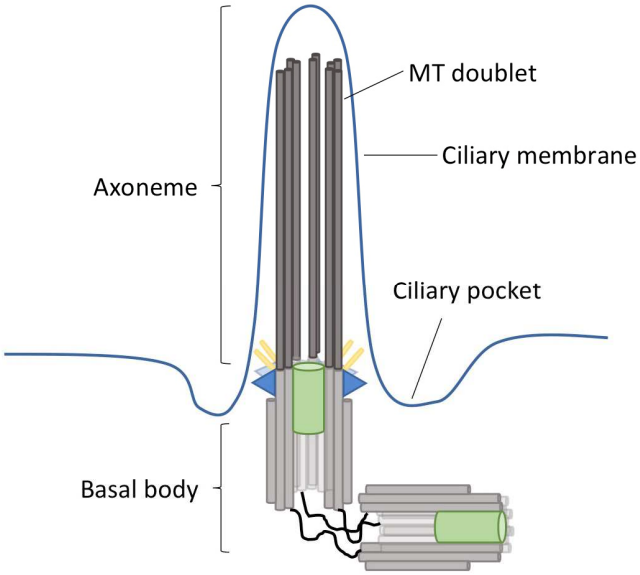


Figure 9. Primary cilia structure. The basal body is anchored at the cellular surface through the distal appendages. The cilium starts growing from the distal part from the mother centriole leading to the formation of the axoneme. This structure is formed by 9 MT doublets evolved by a specialized plasma membrane called ciliary membrane.

Problems on centriole duplication has been recently associated with ciliogenesis defects (Abdelhalim Loukil, 2016). Moreover, although disturbing the γTuRC integrity has been recently associated to defects during centriole duplication (Cota RR, 2016), little is known about the real contribution of γTuRC regulation on ciliogenesis. Probably, this is due to the mitotic defects observed after disrupting the complex. However, it is still unclear deregulation of the γTuRC function, without causing mitotic arrest, has any implication on ciliogenesis. Therefore, it may be interesting to analyses the role γTuRC function during interphase to get a better insight into the contribution of γTuRC regulation in ciliogenesis process.

4) MT nucleation

Under physiological conditions MT nucleation has been shown to be key to organizing MTs into different architectures. Most nucleation events take place at the described MTOCs and are primary mediated by γ-tubulin complexes (Lüders J, Stearns T., 2007*;* Kollman JM, 2011), which work as a template for α- and β- tubulin incorporation (Yixian Zheng, 1995).

4.1) Composition of γ-tubulin complexes

γ-Tubulin was initially described as a new member of the tubulin superfamily, sharing a 33% identity with α-/β-tubulin (Oakley CE & Oakley BR., 1989). When purified from Drosophila, Xenopus and humans γ-tubulin was found to assemble into a ~2.2 MDa complex called γ-tubulin ring complex (γTuRC) (Figure 10). This protein complex is composed of a set of conserved proteins including γ-tubulin, the γ-tubulin complex proteins GCP2-6, GCP-WD/NEDD1 and the more recently identified subunits MZT1 and GCP8/MZT2 (Oegema K, 1999*;* Kollman JM, 2011; Teixidó-Travesa N, 2012, Neus Teixidó-Travesa, 2010). Apart from γ-tubulin, GCPs 2-6 have been described as the core components of the γTuRC (Ruwanthi N. Gunawardane, 2000).They are related to each other and share two conserved regions called Grip motifs. Furthermore, analysis of the crystal structure of GCP4 showed that these proteins are also structurally related, extending the similarities beyond the previously reported grip1 and grip2 motifs (Guillet V, 2011). While the N-terminal conserved region mediates the lateral binding between GCPs, the C-terminal part is involved in the direct binding to γ-tubulin (Knop M & Schiebel E., 1997*;* Geissler S, 1996*;* Kollman JM Z. A., 2008).

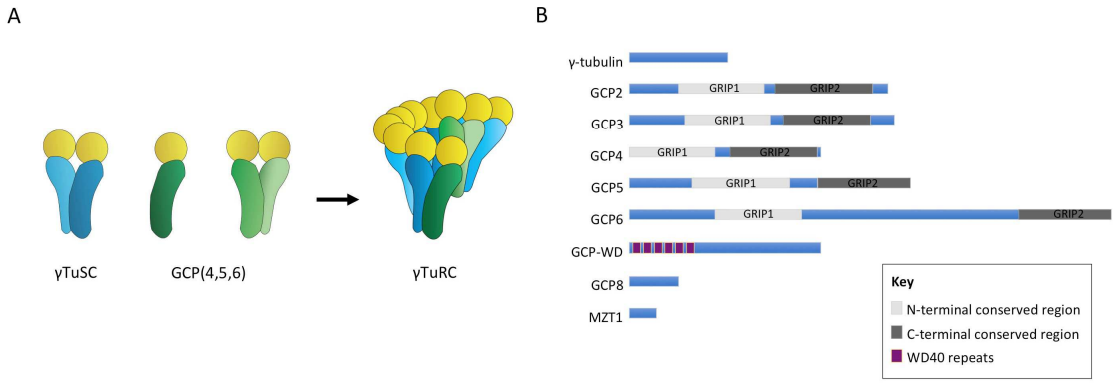


Figure 10. The γTuRC assembly. A) The formation of the γTuRC starts with the assembly of several γTuSC and the following incorporation of GCP4, GCP5 and GCP6, although their exact position in the γTuRC is still unknown. B) Schematic representation of the different γTuRC subunits. Adapted from Teixidó-Travesa et al., 2012.

The other subunits of the complex, GCP-WD/NEDD1, MZT1, and GCP8/MZT2, do not contain grip motifs, but co-purify in similar amounts as GCP2-6. It has been reported by sucrose gradient centrifugation analysis that only the depletion of γ-tubulin or any of the GCPs 2-6 strongly destabilizes the complex in animal cells suggesting that these proteins contribute to γTuRC integrity and that the other subunits may have regulatory roles (Christel Vérollet, 2006; Xiong Y & Oakley BR., 2009; Neus Teixidó-Travesa, 2010). Interestingly, apart from GCP8 depletion, the absence of any γTuRC subunit strongly impairs mitotic progression by affecting spindle assembly and/or function. In the case of NEDD1 and MZT1, which are not required for γTuRC assembly, this effect is due to problems in the targeting of the complex to specific nucleation sites (Lüders J, 2006) and impaired binding of targeting and activation factors to the complex (Cota RR, 2016), respectively.

Apart from nucleating MTs, an additional feature of the γTuRC is that it forces tubulin to form 13-protofilament MT. When polymerized in vitro, the number of protofilaments in MTs varies from 11 to 16 (Chrétien D & Wade RH., 1991; Evans L, 1985). However, in cells most MTs are made of 13 protofilaments.

Studies performed in budding yeast have significantly advanced our understanding of the stoichiometry of the complex since they nucleate MTs through a smaller complex called γTuSC. This 300kDa protein complex is composed of two copies of γ-tubulin and one each

of GCP2 and GCP3. The assembly of seven γTuSC, which is regulated through the phosphorylation of Spc110 (the yeast homologue of pericentrin), leads to the formation of a ring-like structure similar to γTuRC that promotes MT nucleation (Kollman JM, 2011; Lin TC, 2014).

Whereas in mammals an intact γTuRC is required for MT nucleation, it has been shown that in some lower eukaryotes, such as *Schizosaccharomyces pomb*e and *Aspergillus nidulans*, the absence of GCP4, 5 or 6 does not compromise the viability of the organism and only γTuSC is essential (Figure 11) (Andreas Anders & Kenneth E. Sawin, 2011*;* Xiong Y & Oakley BR., 2009).

Homo sapines	Drosophila melanogaster	Arabidopsis thaliana	Aspergillus nidulans	Schizosaccharomyces pombe	Candida albicans	Saccharomyces cerevisiae
γ-tubulin	γ-tubulin	TUBG1/2	mipA	Tug1/Tubg1	Tub1	Tub4
GCP2	Grip84	Spc97p/GCP2	GCP2/B	Alp4	Spc97	Spc97
GCP3	Grip91	Spc98p/GCP3	GCP3/C	Alp6	Spc98	Spc98
GCP4	Grip75	GCP4	GCP4/D	Gfh1	-	-
GCP5	Grip128	GCP5	GCP5/E	Mod21	-	-
GCP6	Grip163	GCP6	GCP6/F	Alp16	-	-
NEDD1/GCP-WD	Grip71	Nedd1	-	-	-	-
MZT1	Mozart1	GIP1a/b	Mzt1	Mzt1	MOZART1	-
GCP8/MZT2	-	-	-	-		-
CD5RAP2, Pericentrin, Myomegalin	Cnn, Plp	-	Pcpa, ApsB	Mto1/Mto2, Pcp1	Spc110, Spc72	Spc110, Spc72
AKAP450/AKAP9	-	-	-	-	-	-
chTOG	Msps	-	Zyg9	Alp14	-	Stu2
TPX2	Mei38	Tpx2	Tpxl-1	-	-	-

Figure 11. Proteins involves in MT nucleation. The table shows orthologous of proteins involves in MT nucleation including the γTuRC, CM1 and/or PACT-domain containing proteins, γTuRC independent proteins.

In addition to its role as a MT nucleation template, the γTuRC has been also described to stabilize the MT by capping the minus end (Andreas Anders & Kenneth E. Sawin, 2011; Y., 2010) and to regulate MT dynamics by limiting the number of catastrophe events (Anaïs Bouissou, 2009).

However, although the γTuRC is widely accepted as the main MT nucleator in cells, recent evidences suggest that other factor may contribute to this activity (Thawani A, 2018). For example, in absence of γTuRC MT density is strongly reduced but the MT network is not completely disrupted, suggesting that other factors can also provide nucleation activity. (Strome S, 2001*;* Sampaio P, 2001*;* Hannak E, 2002*;* Eileen O'Toole, 2012).

4.2) Targeting

Consistent with the variety of MTOCs that exist in different cell types and species, many different proteins have been described that can recruit the γTuRC to specific MTOCs, which in humans include the CDK5 Regulatory Subunit Associated Protein 2 (CDK5RAP2), pericentrin, AKAP450, myomegalin (MMG) and NEDD1 (Figure 11, 13 and 14). Apart from NEDD1 all the other proteins share a small region with similarity to the conserved centrosomin motif 1 (CM1), which was initially described in Drosophila centrosome protein centrosomin/Cnn, required for the binding and activation of the γTuRC (Mikiko Takahashi, 2002*;* Wang Z, 2010*;* Lüders J, 2006). Moreover, pericentrin and AKAP450 share a conserved region at the C-terminus referred to as pericentrin AKAP450 centrosomal targeting (PACT) domain required for the targeting of the γTuRC to centrosomes (Alison K. Gillingham & Sean Munroa, 2000). However, beyond having a role at the centrosome, the function of AKAP450 has been better studied at the Golgi apparatus. There, together with the cis-Golgi-associated protein GM130, it is involved in the recruitment of the γTuRC at this organelle (Figure 14) (Sabrina Rivero, 2009*;* Martin M & Akhmanova A, 2018). In contrast, NEDD1 does not contain any of the mentioned motifs/domains but nevertheless can also bind and target the γTuRC to the centrosome through its C-terminus and N-terminus, respectively (Haren L, 2006).

The overlapping function of these proteins suggests an additional mechanism of control that specifically regulates their activity. Indeed, most of these proteins are known to be regulated by phosphorylation and in some cases these modifications were shown to promote targeting of the γTuRC to specific MTOCs (Pinyol R, 2013*;* Gomez-Ferreria MA, 2012, Zhang X, 2009*;* Weronika E. Borek, 2015).

4.3) Activity

Although MT nucleation takes place at MTOCs, only about 1% of the total γ-tubulin is concentrated at the centrosome, suggesting that most of the free cytosolic fraction of γTuRC is inactive (Moudjou M, 1996; Bauer M, 2016). Thus, apart from proteins that mediated targeting, other factors may be required for the specific regulation of γTuRC nucleation activity (Figure 12).

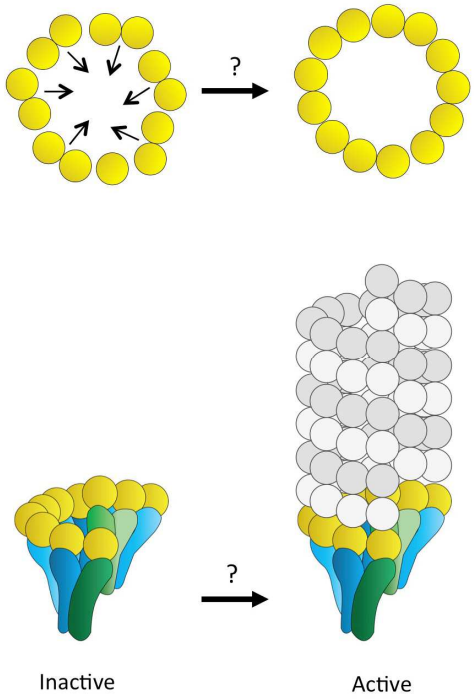


Figure 12. Schematic representation of γTuRC activation. The assembly of the γTuRC is not sufficient to trigger MT nucleation. Additional mechanism, including binding of proteins directly involved in the activation of the complex or that might induce a conformational change to the γ-tubulin disposition are required to stimulate γTuRC nucleation.

4.3.1) Regulation at the level of γTuRC

MT nucleation in budding yeasts, which is the simplest system to study this process, requires the oligomerization of the γTuSC through the binding of two adaptor proteins, Spc110 and Spc72, which contain an Spc110/Pcp1 motif (SPM) and/or a centrosomin motif 1 (CM1) (Lin TC N. A., 2015). Oligomerization leads to the formation of an efficient nucleation template, overcoming the limited nucleation activity of a single γTuSC (Kollman JM, 2011; (Kollman JM, 2008). Curiously, studies performed in fission yeast reveal that,

although they contain the specific γTuRC subunits GCP4, GCP5 and GCP6, these are not required for MT nucleation. In fact, similar to budding yeast, the CM1 containing protein complex Mto1/Mto2 is sufficient to promote γTuSC oligomerization and stimulate MT nucleation (Lynch EM, 2014). Recently, it has been shown *that Candida albicans*, an infectious fungus, has the same set of proteins for MT nucleation as the budding yeast *Saccharomyces cerevisiae* (γTuSC and the adaptor proteins Spc110 and Spc72) but additionally contains the small protein MZT1. In this organism MZT1 is required for both γTuSC assembly and MT nucleation by mediating the binding of other key adaptor proteins (Cota RR, 2016; Lin TC1, 2016). However, in higher eukaryotes γTuRC, which can be considered equivalent to γTuSC oligomers in yeast, is not sufficient to stimulate MT nucleation, suggesting more complex regulation. In this case, similar to the targeting, the CM1 and/or PACT domain containing proteins are also fundamental to stimulate MT nucleation (Figure 13) (Lin TC N. A., 2014; Choi YK, 2010). Due to the presence of several proteins the contribution of each one varies according to the different MTOCs. Thus, while CDK5RAP2 and pericentrin function mainly at the centrosome, AKAP450 and MMG play a more important role at the Golgi apparatus (Figure 13 and 14) (Gavilan MP, 2018*;* Wu J d. H., 2016*;* Roubin R, 2013).

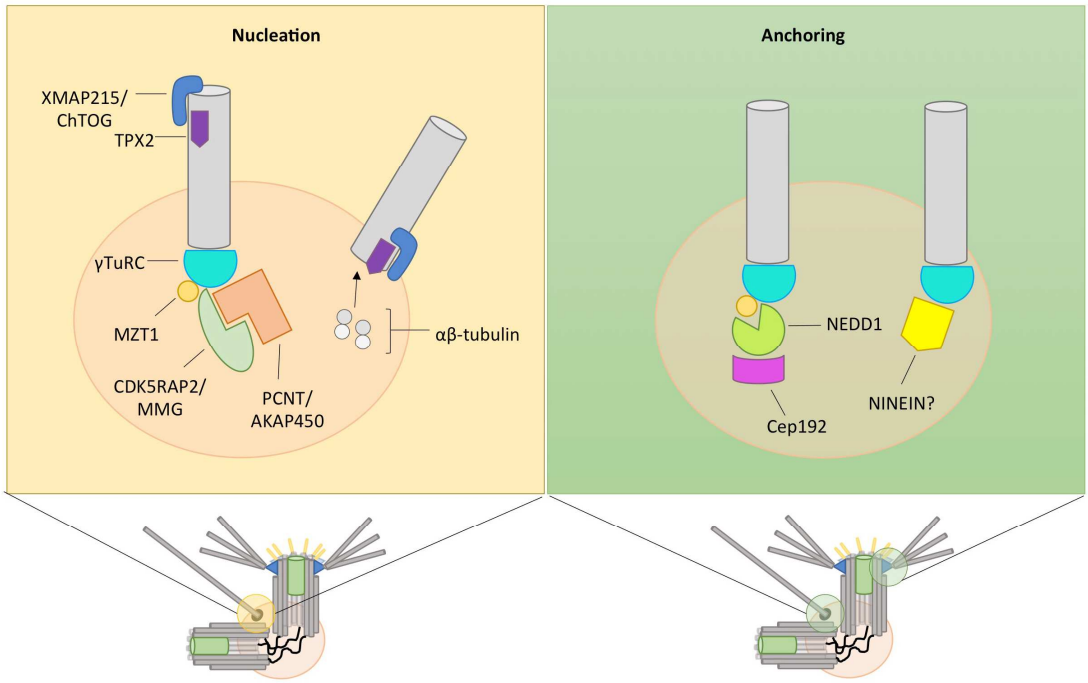


Figure 13. MT organization at the centrosome. MT nucleation at the centrosome is regulated by different mechanism. The γTuRC is recruited and regulated by several proteins, including the CDK5RAP2, MMG, PCNT and AKAP450. Among them, CDK5RAP2 has been shown to bind to the complex through MZT1 and "activate" γTuRC nucleation. Moreover, TPX2 and XMAP215/ChTOG are proposed to stimulate MT nucleation and elongation in a γTuRC independent manner. Apart from nucleation centrosome are also involved in MT anchoring. NEDD1 has been shown to be indispensable for targeting the complex to the centrosome but is not required for its nucleation activity. Cep192 binds NEDD1 and regulates its phosphorylation status during mitosis (*Gomez Ferreria MA et al., 2012*). The centrosome sub-distal appendage protein Ninein is involved in MT attachment although the mechanism that regulate this process is still not clear.

Remarkably, although nucleation activity at this organelle is regulated by several mechanisms, the large scaffolding protein AKP450 has been shown to play a pivotal role. It is involved in the recruitment of the γTuRC, CDK5RAP2 and MMG to the Golgi by its binding to Golgi matrix protein GM130 (Figure 14). In addition, AKAP450, together with MMG, forms a complex that regulates MT minus end stabilization at the Golgi by binding to CAMSAP2 and EB1/EB3. Moreover, AKAP450 can bind CLASPs proteins, which were suggested to regulate both MT nucleation and MT minus end stabilization at the Golgi. Finally, MTCL1, a MAP, binds AKAP450 and CLASP and contributes to MT tethering at this organelle (Figure 14) (Yang C1, 2017*;* Wu J d. H., 2016; Martin M & Akhmanova A, 2018*;* Petry S & Vale RD, 2015).

γTuRC activating function has been also proposed for other proteins that contain neither CM1 nor PACT domains, such as NME7 or Ninein-like protein (NINL) (Casenghi M, 2003; Liu P1, 2014).

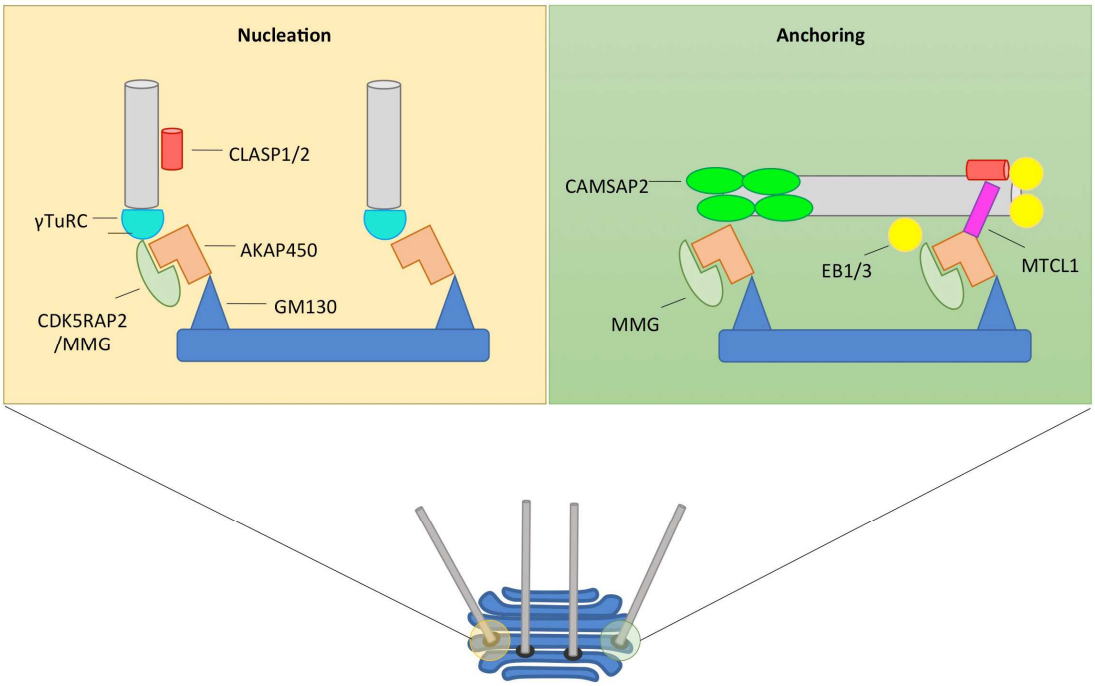


Figure 14. MT organization at the Golgi apparatus. The cis-Golgi protein GM130 plays a pivotal role in the regulation of MT organization at this organelle by recruiting AKAP450, which in turn stimulate MT nucleation by directly binding to the γTuRC and recruiting CDK5RAP2 and MMG. Additionally, together with MMG, AKAP450 participates in the anchoring of free MT by binding the minus-end stabilizing factor CAMSAP2. MMG is also involved in the tethering of CAMSAP2-decorated MTs by recruiting EB1 and EB3. Other proteins, such as MTLC1 and CLASP1/2, participate in the anchoring and/or nucleation of MT at the Golgi apparatus.

In conclusion, while several factors that stimulate γTuRC nucleation activity have been identified, the underlying mechanism has not yet been revealed. However, structural work has suggested the requirement for a conformational change in γTuRC to generate an efficient nucleation template (Kollman JM, 2011; Kollman JM P. J., 2010).

4.3.2) Regulation at the level of the nascent MT

Studies on the regulation of MT nucleation activity have been typically focused on the regulation of γTuRC itself. However, recent work suggests that additional factors that do not modify the γTuRC-template but act on the nascent MT may contribute to the regulation of MT formation in the cell (Figure 15).

Several MT associated proteins (MAPs) have been proposed to have a role in MT nucleation. TPX2 has been a recurrent candidate since the depletion of this protein abolishes chromatin dependent MT nucleation in mitotic Xenopus egg extract (Gruss OJ, 2001*;* Petry S, 2013). In fact, in vitro experiments proved that TPX2 can stabilize the MT minus end and synergistically with the MT polymerase XMAP215, or its human homologue chTOG/CKAP5, stimulates MT nucleation (Roostalu J & Surrey T, 2017). In addition, more recent advances in this field suggest that XMAP215 binds directly to γTuRC to stimulate MT nucleation in vitro by promoting the polymerization of tubulin subunits on the γTuRC template (Thawani A, 2018).

Single-molecule assays have been recently used to analyze the effects of several catastrophe factors on the capacity of MTs to grow from nucleation templates. Interestingly, EB1 and MCAK were reported to inhibit MT nucleation through their MT catastrophe promoting activity and one can speculate that other members of the kinesin-13 family may have similar effects (Wieczorek M, 2015). The kinesin-13 family comprises 4 members named KIF2A, KIF2B, KIF2C/MCAK and KIF24 (Walczak CE, 2013). Despite belonging to the superfamily of motor proteins that move along the MT lattice, kinesin-13 members typically localize at MT ends where they promote MT depolymerization in an ATP-dependent manner. Kinesin-13 family members are required for a wide variety of functions, such as spindle assembly, chromosome segregation and axonal growth (Ganem NJ & Compton DA., 2004; Homma N, 2003; Jang CY & Fang G, 2009; Hood EA, 2012). Moreover, both KIF24 and KIF2A localize at the centrosome and regulate ciliogenesis, either by stabilizing the primary cilia inhibitory protein CP110 or by impairing the disassembly of the primary cilia after cell cycle re-entering respectively (Kobayashi T, 2011*;* Tatsuo Miyamoto K. H., 2015).

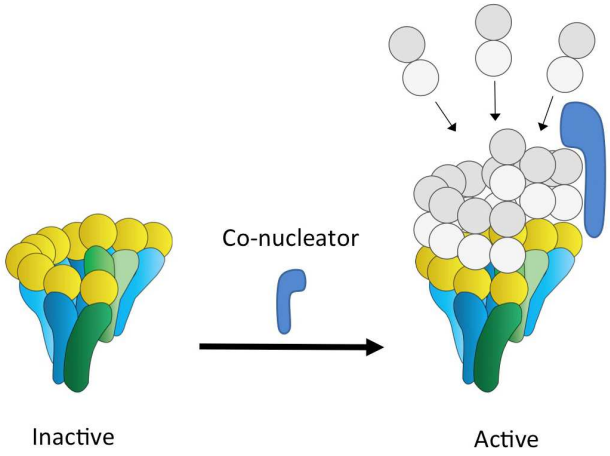


Figure 15. Activation of nucleation at the levels the nascent MT. MT nucleation activity can be regulated by the action of co-nucleator, which may stabilize MTs and promote its elongation of early intermediates.

Consistent with its important cellular roles KIF2A has been implicated in a variety of human diseases ranging from cancer to brain malformation and neuronal disorders such as schizophrenia, epilepsy and eye defects (Zhao P, 2018; Xie T, 2018; Homma N Z., 2018).

Additionally, independent experiments performed in cell lines described the DNA polymerase δ subunit (PolD1) as a negative regulator of the γTuRC nucleation activity (Shen Y, 2017)

Altogether, these findings suggest that MT formation is a highly regulated process that is still not completely understood.

5) The γTuRC subunit GCP8/MZT2

GCP8 was identified, together with MZT1, by mass spectrometry analysis of two independent γ-tubulin purification experiments (Hutchins J ..., 2010; Neus Teixidó-Travesa, 2010). GCP8 is a small protein (around 18kDa) encoded majorly by a single gene. GCP8-related sequences are not found in fungi or plants but are present in most deuterostomes (Figure 16). Curiously, in humans, but not other mammals, two distinct genes encode GCP8 (GCP8A/GCP8B), although their protein product is more than 95% identical, suggesting relatively recent gene duplication. Interestingly, the most conserved region of GCP8 is in its

N-terminal part, which is predicted to be rich in helical secondary structures and implicated in the binding of GCP8 to the γTuRC (unpublished work from the Lüders lab).

Figure 16. The γTuRC subunit GCP8. GCP8 amino acid sequence alignment between different species **(Neus Teixidó-Travesa, 2010)**.

As I commented before, the depletion of GCP8 does not affect the stability of the γ-TuRC, suggesting that it does not participate in forming the γTuRC structure. However, GCP8 depletion slightly reduces MT nucleation by impairing the binding of other γTuRC components at the centrosome during interphase. Further, in contrast to all other γTuRC subunits, GCP8 deficiency does not seem to affect mitotic spindle assembly and progression (Neus Teixidó-Travesa, 2010). However, beyond the relatively mild interphase-specific phenotype, little is known about the cellular role of GCP8 and its role as γTuRC regulator.

Unpublished data from our laboratory indicates that GCP8 may also regulate MT dynamics. Both, depletion or overexpression of GCP8 enhances the polymerization rate of MTs by an unknown mechanism. Additionally, changes in the expression level of GCP8 in cell lines reduce the ability of cells to adhere to substrate, changes cell morphology (more roundish shape with membrane blebbing) and regulates migration capacity. Interestingly, we identified by immunofluorescence microscopy an undescribed localization of GCP8 with actin filaments at the cell cortex and near focal adhesions. Therefore, GCP8 could have a role in mediating crosstalk between the MT and the acto-myosin network.

OBJECTIVES

Based on previous observations in the Lüders laboratory I focused my PhD thesis on the following objectives:

I) Analyse how GCP8 binds to the γTuRC

II) Characterize the interaction of GCP8 with other proteins

III) Determine how GCP8's role at the centrosome and potentially at non-centrosomal MT organizing centers affects cell function

MATERIALS AND METHODS

Cloning and plasmids

Full-length GCP8B was PCR amplified from a human liver cDNA library using the following primers: CCGCTCGAGCGATGGCGGCGCAGGGCGTAGG and CCGGAATTCCTAGGTGCTGCCCTGCGTAGGGCT.

For expression in human cells amplified GCP8 sequences were inserted into pEGFP-C1 (Clontech, Palo Alto, CA) containing a modified multiple clone sites using *FseI* and *AscI* restriction sites. GFP-GCP8 was sub-cloned into a pCS2+N-FLAG (Addgene) and pCS2+HA using *FseI* and *AscI* restriction sites. GFP-GCP8 and FLAG-GCP8 siRNA resistant was generated by site directed-mutagenesis (following the recommendation of QuickChange Site-Directed Mutagenesis). Also, full length GCP8 was amplified by PCR with the following primers: 5´-GGCGCGCCAATGGCGGCGCAGGGCGT-3´, 5´-GCGGCCGCCTAGGTGCTGCCCTGCGT-3´, 5´-GGTACCCAAATGGCGGCGCAGGGCGTA-3´and 5´-GGCGCGCCAGGTGCTGCCCTGCGT AG-3 and inserted into the plasmids pCDNA/FRT/TO-hBirAm and pCDNA/FRT/TO-hBirAm-bTRC respectively.

The following fragments of the N-terminal domain of GCP2-6 were PCR amplified and cloned in pCS2+N-FLAG using *FseI* and *AscI* restriction sites: GCP2 (1-506), GCP2 (1-217), GCP2 (217-506), GCP2 (1-125), GCP2 (24-506), GCP2 (50-506), GCP2 (125-506), GCP3 (1-552), GCP4 (1-347), GCP5 (1-713), GCP6 (1-710). Other plasmids used in this study were FLAG-GCP2 full length, FLAG-GCP2Δ24 (24-903), GFP-GCP8 Nter (1-111), GFP-GCP8 Cter (112-158), GFP-KIF2A (a gift from Gotha Goshima Laboratory).

Cell culture and treatments

Human RPE-1 cells were cultured in DMEM/F-12 mixture medium (1:1; 11330-032, Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37°C with 5% CO2. HEK293, U2OS and HeLa cells were cultured in DMEM medium (Thermo Fisher Scientific) with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Cells were transfected with plasmid using calcium phosphate or Lipofectamine 2000 (Invitrogen) according to manufactures protocol. For knocking-down (KD) target genes cells were transfected with 100nM siRNA using LipoRNAimax (Invitrogen). For RNAi mediated depletion the sense sequences are the

following: GCP8_oligo1 5´-CGACGUGUUCAAGAUCCUGtt-3´ (Ambion, ID s198094), GCP8_oligo2 5´-GCAAAGGCUACAUGUUACCtt-3´ (Ambion, ID 279234), GCP8_oligo3 5´-CGCGAAGGAUCCAGCCAGA-3´ (Sigma, ID SASI_Hs02_00357687), GCP8_oligo4 5´-GAGAUGGAGCUGAUCGAGCtt-3´ (Ambion, ID s228552), GCP8_oligo5 5´-CCGAGGAGAUGGAGCUGUA-3´ (Dharmacon, ID 80097), GCP8_oligo6 5´-TACCTCCTTCAGTTGATAATA-3´ (Qiagen, ID SI05017271), GCP2 5´-GGCUUGACUUCAAUGGUUUtt-3´ (Ambion, ID s21286), KIF2A 5'-GGCAAAGAGAUUGACCUGG-3' (described at Jianli Wang, 2014)

To perform microtubule regrowth, dishes containing poly-d-lysine-coated coverslips with U2OS or RPE-1 cells were incubated for 30 min in an ice-water bath to depolymerize microtubules. Microtubule regrowth was initiated at 37°C and proceeded for various time periods, followed by PFA 4% fixation. For analyzing MT nucleation at the Golgi, RPE1 cells were first treated with 2,5µg/ml for 3 hours at 37°C and, after two washes with cold PBS, cells were treated in the same way I mentioned before.

To generate the T-REx HeLa stable cell lines expressing FLAG-BirA or FLAG-BirA-GCP8 fusion protein cells were co-transfected with a pcDNA5 FRT/TO FLAG-BirA or pcDNA5 FRT/TO FLAG-BirA-GCP8 expression plasmid together with the flp-recombinase expression plasmid pOG44 in a 1:2 proportion. Transfected cells were maintained in DMEM medium (Thermo Fisher Scientific) with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin and selected with hygromycin B (200µg/ml). Resistant clones were tested for expression of the tagged proteins.

Other stable cell lines generated in this study were U2OS and RPE-1 stably expressing GFP and GFP-GCP8. Cells were transfected with GFP and GFP-GCP8 expression plasmids and selected in the presence of 0.4µg/ml geneticin.

Antibodies

Anti-GCP8, anti-GCP2, anti-GCP3, anti-GCP4, anti-GCP5, anti-GCP6 antibodies were produces as described in *Teixido et al 2010* and *R.R. Cota et al. 2017*.

Other antibodies used in this study: mouse anti-γ-tubulin (GTU-88, Sigma), mouse anti-γ-tubulin (Exbio, Prague, Czech Republic), mouse anti-α-tubulin (DM1A, Sigma), rabbit anti-α-tubulin (18251, Abcam) rabbit anti-GCP-WD/NEDD1 (*Lüders et al., 2006*), rabbit anti-pericentrin (*Lüders et al., 2006*), mouse anti-acetylated tubulin (T6793, Sigma), mouse anti-GFP (3E6, A11120, Life Technologies), rabbit anti-GFP (Torrey Pines Biolabs, Houston, TX), rabbit anti-GFP (A6455, Life Technologies), mouse anti-GM130 (ab1299, Abcam), rabbit anti-Ki67 (NB110-89717, Bio-techne), mouse anti-Centrin 3 (04-1624, Merck Millipore), mouse anti-FLAG (F3165, Sigma-Aldrich), rabbit anti-FLAG (F7425, Sigma) mouse anti-GAPDH (sc-47724, Santa Cruz Biotechnology, INC), rabbit anti-KIF2A (NB500-180, Novusbio), rabbit anti-Cep170 (A301-024A, Bionova), mouse anti-Cep170 (41-3200, Life Technologies), mouse anti-Ninein (MABT29, Merck Millipore), mouse anti-vinculin (V9264, Sigma), rabbit anti-phospho-S6 ribosomal protein (#5364, Cell Signalling), rabbit anti-p27 Kip1 (PA5-27188, Invitrogen), mouse anti-YAP (sc-101199, Santa Cruz Biotechnology), Alexa 488-phalloidin (A12379, Life technologies), phalloidin-TRITC (P1951, Sigma), Alexa 568-streptavidin (Invitrogen S-11227), Alexa 350- 488- and Alexa 568-conjugated secondary antibodies used for immunofluorescence microscopy were from Invitrogen (Carlsbad, CA), and peroxidase-coupled secondary antibodies for western blotting were from Jackson Immunoresearch Laboratories (West Grove, PA).

Immunoprecipitation and Western blotting

HEK293 and HeLa cells were lysed 10 min on ice in lysis buffer (50mM HEPES at pH 7.4, 150 mM NaCl, 1mM $MgCl_2$, 1mM EGTA, 1% NP40) and supplemented with 1x protease inhibitors. After centrifugation for 15 min at 16,000g at 4°C cleared lysates were incubated with specific antibodies for 1 hours at 4°C and sepharose protein G beads (Sigma, P3296-5ML) or dynabeads protein G (Sigma, 10003D) were added and the mixture was incubated for an additional hour. The beads were pelleted and washed three times with the corresponding lysis buffer. Samples were prepared for SDS-PAGE

by boiling in 1x sample buffer (83mM Bis-Tris, 50mM HCl, 3.3% glycerol, 1.3% SDS, 0.3mM EDTA, 0.01% bromophenol blue, 0.83% β-mercaptoethanol). Samples were loaded on Bis-Tris acrylamide gels (4% for stacking and 10% for separation) and run at 120mV in 1x MOPS buffer (50 mM MOPS, 50 mM Tris-base, 0.1 %SDS, 1 mM EDTA) or MES buffer (50 mM MES, 50 mM Tris, 0.1% SDS, 1 mM EDTA). Proteins were transferred to membranes for 1 hour at 90V in 1x transfer buffer (25 mM Tris, 192 mM Glycine, 20% methanol). Membranes were blocked in 1x TBS (25 mM Tris, 150 mM NaCl, and 2 mM KCl) + 0.1 % Tween20 + milk (5%) and probed with antibodies diluted in 1x TBS-T + milk (5%). Membranes were washed with TBS-T between each incubation step.

For KIF2A Immunoprecipitation cells were lysed with a different lysis buffer containing (50mM HEPES ph 8.0, 100mM KCl, 2mM EDTA, 0,1% NP40, 10% glycerol, and 1x protease inhibitors).

For FLAG-BirA-GCP8 IP the cell pellet was weighed, and 1:4 pellet weight:lysis buffer (by volume) was added. Lysis buffer consisted of 50 mM HEPES-NaOH (pH 8.0), 100 mM KCl, 2 mM EDTA, 0.1% NP40, 10% glycerol, 1 mM PMSF, 1 mM DTT and 1:500 protease inhibitors cocktail (Sigma-Aldrich, St. Louis, MO). On resuspension, cells were incubated on ice for 10 min, subjected to one additional freeze–thaw cycle, then centrifuged at 27000 × g for 20 min at 4°C. Supernatant was transferred to a fresh 15 ml conical tube, and 1:1000 benzonase nuclease (Novagen), plus 30 µl packed equilibrated Flag-M2 agarose beads (Sigma-Aldrich) were added. The mixture was incubated for 2 h at 4°C with end-over-end rotation. Beads were pelleted by centrifugation at 1000 × g for 1 min and transferred with 1 ml of lysis buffer to a fresh centrifuge tube. Beads were washed once with 1 ml lysis buffer and twice with 1 ml ammonium bicarbonate (ammbic) rinsing buffer (50 mM ammbic, pH 8.0, 75 mM KCl). Elution was performed by incubating the beads with 150 µl of 125 mM ammonium hydroxide (pH >11). The elution step was repeated twice, and the combined eluate centrifuged at 15000 × g for 1 min, transferred to a fresh centrifuge tube. The sample was lyophilized and resuspended in buffer A (0.1% formic acid). 1/5th of the sample was analyzed per MS run.

For the Biotin-streptavidin affinity purification the cell pellet was resuspended in 10 mL of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton

X-100, 0.1% SDS, 1:500 protease inhibitor cocktail (Sigma-Aldrich), 1:1000 benzonase nuclease (Novagen), incubated on an end-over-end rotator at 4°C for 1h, briefly sonicated to disrupt any visible aggregates, then centrifuged at 16,000*g* for 30 min at 4°C. The supernatant was transferred to a fresh 15 mL conical tube, 30 µL of packed, pre-equilibrated streptavidin-sepharose beads (GE) were added, and the mixture incubated for 3h at 4°C with end-over-end rotation. Beads were pelleted by centrifugation at 2000 rpm for 2 min and transferred with 1 mL of lysis buffer to a fresh Eppendorf tube. Beads were washed once with 1 mL lysis buffer and twice with 1 mL of 50 mM ammonium bicarbonate (pH 8.3). Beads were transferred in ammonium bicarbonate to a fresh centrifuge tube and washed two more times with 1 mL ammonium bicarbonate buffer. Tryptic digestion was performed by incubating the beads with 1 ug MS grade TPCK trypsin (Promega, Madison, WI) dissolved in 200 µL of 50 mM ammonium bicarbonate (pH 8.3) overnight at 37°C. The following morning, an additional 0.5 µg trypsin was added, and the beads incubated 2h at 37°C. Beads were pelleted by centrifugation at 2000*g* for 2 min, and the supernatant was transferred to a fresh Eppendorf tube. Beads were washed twice with 150 µL of 50 mM ammonium bicarbonate, and these washes were pooled with the first eluate. The sample was lyophilized and resuspended in buffer A (0.1% formic acid). 1/5th of the sample was analyzed per MS run.

Mass spectrometry analysis

High performance liquid chromatography was conducted using a 2cm pre-column (Acclaim PepMap 50 mm x 100 um inner diameter (ID)), and 50 cm analytical column (Acclaim PepMap, 500 mm x 75 um diameter; C18; 2 um; 100 Å, Thermo Fisher Scientific, Waltham, MA), running a 120 min reversed-phase buffer gradient at 225 nl/min on a Proxeon EASY-nLC 1000 pump in-line with a Thermo Q-Exactive HF quadrupole-Orbitrap mass spectrometer. A parent ion scan was performed using a resolving power of 60,000, then up to the twenty most intense peaks were selected for MS/MS (minimum ion count of 1,000 for activation), using higher energy collision induced dissociation (HCD) fragmentation. Dynamic exclusion was activated such that MS/MS of the same *m*/*z*

(within a range of 10 ppm; exclusion list size = 500) detected twice within 5 sec were excluded from analysis for 15 sec. For protein identification, Thermo .RAW files were converted to the .mzXML format using Proteowizard [18606607], then searched using X!Tandem [14976030] and Comet [23148064] against the human Human RefSeq Version 45 database (containing 36113 entries). Search parameters specified a parent ion mass tolerance of 10 ppm, and an MS/MS fragment ion tolerance of 0.4 Da, with up to 2 missed cleavages allowed for trypsin. Variable modifications of +16@M and W, +32@M and W, +42@N-terminus, and +1@N and Q were allowed. Proteins identified with an iProphet cut-off of 0.9 (corresponding to ≤1% FDR) and at least two unique peptides were analyzed with SAINT Express v.3.3. Thirteen control runs for BioID and 6 control runs for FLAG-IP (from cells expressing the FLAG-BirA* epitope tag) were collapsed to the two highest spectral counts for each prey and compared to the two biological and technical replicates of N- and C-ter FLAG-BirA-tagged GCP8 BioID and FLAG-IP. High confidence interactors were defined as those with a SAINT score>0.8 (corresponding to a FDR<1%).

Fluorescence microscopy

HeLa, U2OS and RPE-1 cells grown on coverslips were fixed in methanol at −20°C or PFA 4% at RT for at least 10 min and processed for immunofluorescence. Fixed cells were blocked in PBS-BT (1x PBS, 0.1% Triton X-100, and 3% BSA) and incubated with primary and then secondary antibodies in the same buffer. Images were acquired with an Orca AG camera (Hamamatsu) on a Leica DMI6000B microscope equipped with 1.4 NA 63x and 100x oil immersion objectives. AF6000 software (Leica) was used for image acquisition. For further image processing and quantification of fluorescence intensities ImageJ software was used. Intensities were measured in images acquired with constant exposure settings and were background-corrected.

FACS analysis

RPE-1 were transfected with GCP8 and KIF2A siRNAs for 72h and then prepared for flow cytometry by fixation in 70% ethanol. To measure DNA content, cells were stained with propidium iodide (Sigma). Fluorescence was measured by flow cytometry using a Coulter XL Flow Cytometer (Flow Cytometer Unit, Scientific and Technical Services University of Barcelona).

Statistical analysis

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

RESULTS

1) Mapping the γTuRC binding site to GCP8

To gain mechanistic insight into the regulation of γTuRC by GCP8 I decided to study the GCP8-γTuRC interaction. Previously, in unpublished results from our laboratory, we observed that GCP8 binds to the γTuRC through its N-terminal region but the binding partner in γTuRC remained unknown.

1.1) GCP8 binds to the N-terminal region of GCP2

In order to describe the binding sites between GCP8 and the γTuRC I co-transfected HEK293 cells with GFP-tagged GCP8 and deletion mutants of each GCP fused to a FLAG-tag. I pulled down the FLAG-tagged proteins with immobilized anti-FLAG antibodies and then probed the samples by western blot (WB). Note that for this assay I used the N-terminal fragment of GCP2-6 since the C-terminal region is involved in the binding of γ-tubulin. The result clearly shows that GCP8 only co-immunoprecipitates with the N-terminal GCP2 construct (Figure 17), and this interaction was also confirmed by a yeast-two-hybrid assay in collaboration with the Roig Lab (IBMB-CSIC, Barcelona, Spain; data not shown).

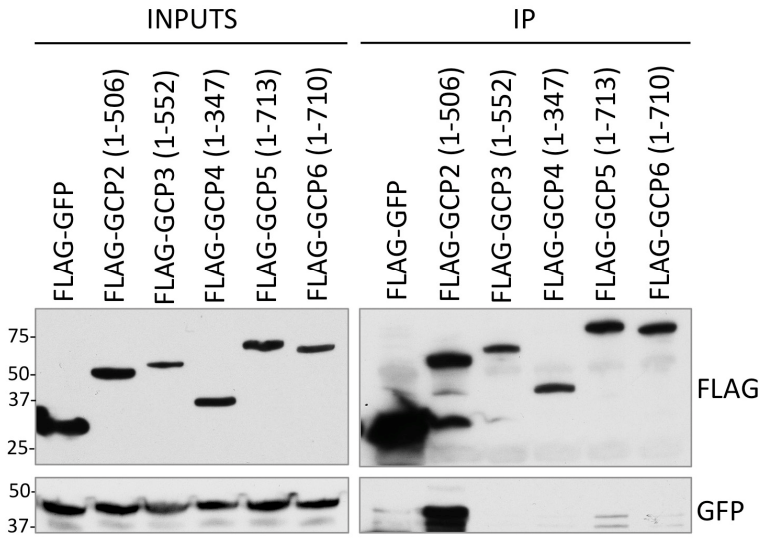


Figure 17. GCP8 interaction with γTuRC subunits. Immunoblot for FLAG and GFP from inputs and immunoprecipitated lysates after co-expression of EGFP-GCP8 with FLAG-tagged N-terminal fragments of GCP2-6.

1.1.1) The first 24 amino acids of GCP2 are required for binding to GCP8

To define the interaction between GCP8 and GCP2 in more detail I generated different GCP2 deletion constructs fused to a FLAG tag. Following the same strategy as in the previous experiment I found that the first 24 amino acids of GCP2 are required for the binding of GCP8 to the complex (Figure 18A-B).

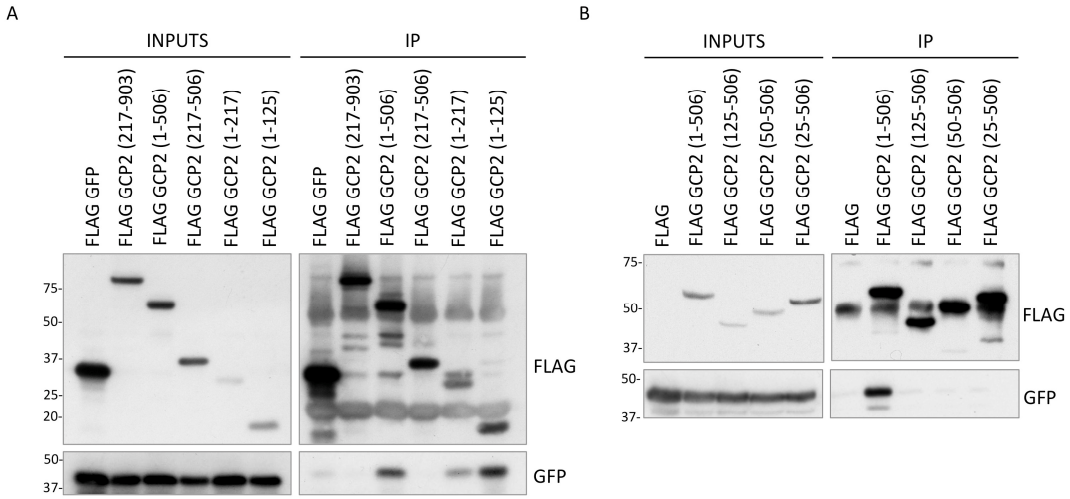


Figure 18. Mapping of the GCP8 binding region in GCP2. A-B) Immunoblot for FLAG and GFP from inputs and immunoprecipitated lysates after co-expression of EGFP-GCP8 with FLAG-tagged GCP2 deletion mutants.

1.2) The GCP2 (25-903) mutant assembles into γTuRC but is defective in GCP8 binding

To test if full length GCP2 lacking the first 24 (GCP2Δ24**)** amino acids was also defective in GCP8 binding, I compared the binding capacity between wild type and mutant GCP2 (note that I included fragment GCP2 (1-125) that binds GCP8 but does not integrate into γTuRC as an additional control). The results showed that, although both constructs can pull down γ-tubulin and GCP4 with similar efficiency, the binding of endogenous GCP8 to the GCP2Δ24 construct is clearly impaired (Figure 19A).

Moreover, in order to analyze whether the GCP2 mutants may work in a dominant-negative manner, I analyzed the ability of endogenous GCP8 to associate with γTuRC in cells overexpressing wild type GCP2 or mutants by pulling down the endogenous GCP8 and probing for γTuRC subunits. I found that the GCP2Δ24 mutant only slightly reduced the interaction of GCP8 with γTuRC (Figure 19B). Expression of the shorter GCP8-binding fragment had no obvious effect on association of endogenous GCP8 with γTuRC.

Interestingly, consistent with their ability to bind GCP8, I also noticed that expression of wild type GCP2 or the GCP8-binding fragment increased the levels of endogenous GCP8 in the input lysates, whereas the GCP2Δ24 mutant caused a slight decrease.

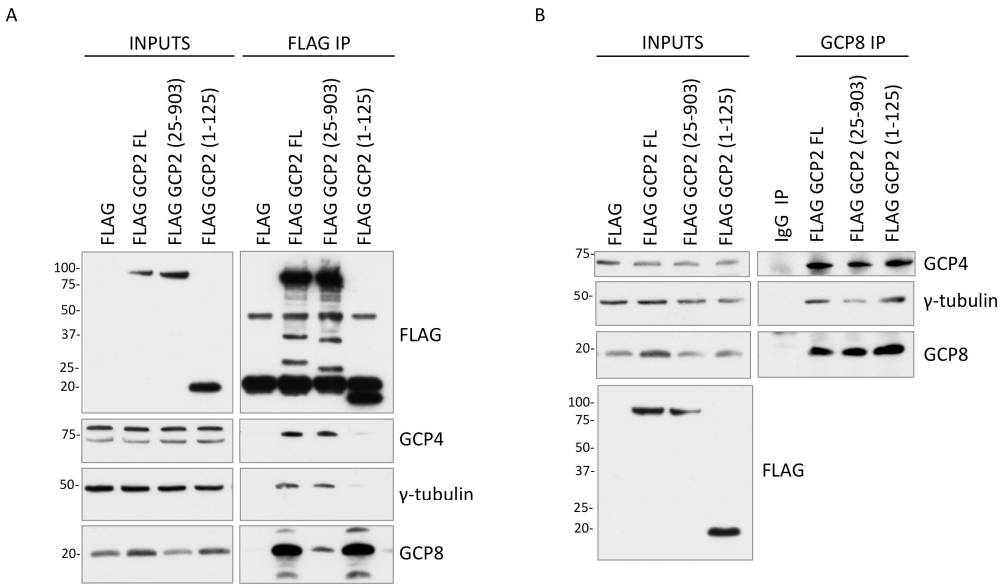


Figure 19. Effects of GCP2Δ24 on interaction of endogenous GCP8 with γTuRC. A) Immunoblot for FLAG, GCP4, γ-tubulin, GCP8 from inputs and immunoprecipitated lysates of cells expressing FLAG-tagged GCP2 constructs. B) Immunoblot for FLAG, GCP4, γ-tubulin, GCP8 from inputs and lysates after immunoprecipitation with GCP8 antibody from cells expressing FLAG-tagged GCP2 constructs.

As we described, in contrast to other γTuRC subunits, GCP8 depletion does not compromise mitotic progression in cancer cell lines (Neus Teixidó-Travesa, 2010). For my project I decided to study the role of GCP8 in a non-cancer cell line. I chose RPE-1 cells, a non-transformed, telomerase-immortalized human retinal epithelial cell line.

To complement the immunoprecipitation experiments I analyzed whether the expression of the GCP2Δ24 mutant affects the centrosomal localization of GCP8. As one can see in the immunofluorescence (IF) images, both the wild type and the mutant constructs localize at the centrosomes but the levels of GCP8 were significantly reduced in cells expressing the mutant, suggesting that GCP8 is recruited to the centrosome through binding to the N-terminus of GCP2 (Figure 20A and B).

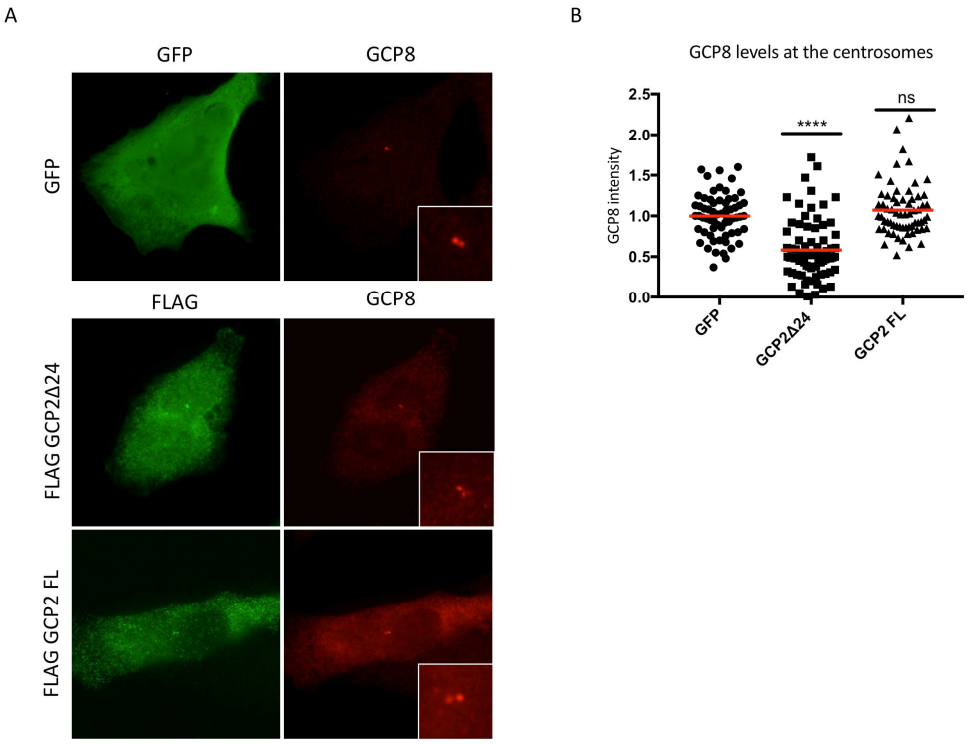


Figure 20. GCP8 centrosome localization after GCP2Δ24 overexpression. A) RPE-1 cells were transfected with GFP, FLAG GCP2Δ24 or FLAG GCP2 FL (full length). Cells stained with GFP, FLAG and GCP8 antibodies are shown. B) The fluorescence intensity of centrosomal signal was quantified for GCP8 and individual values were plotted as normalized values relative to the control (n>60 cells from three independent experiments, ns: not significant; ****p<0.0001)

2) GCP8 depletion changes cell morphology

To directly evaluate the function of GCP8, I knocked down GCP8 levels by using small interfering RNA (siRNA). Interestingly, I observed that the morphology of cells changed dramatically in absence of GCP8: cells appeared smaller and more rounded compared to controls. Indeed, when I quantified the overall area covered by the cells I observed a significant reduction after depletion of GCP8 (Figure 21A-C). I observed the same phenotype with two different oligos, which diminishes the probability of this being an off-target effect. It is worth mentioning that although the morphology in RPE-1 cells also changes, the quantifications shown were done in U2OS cells.

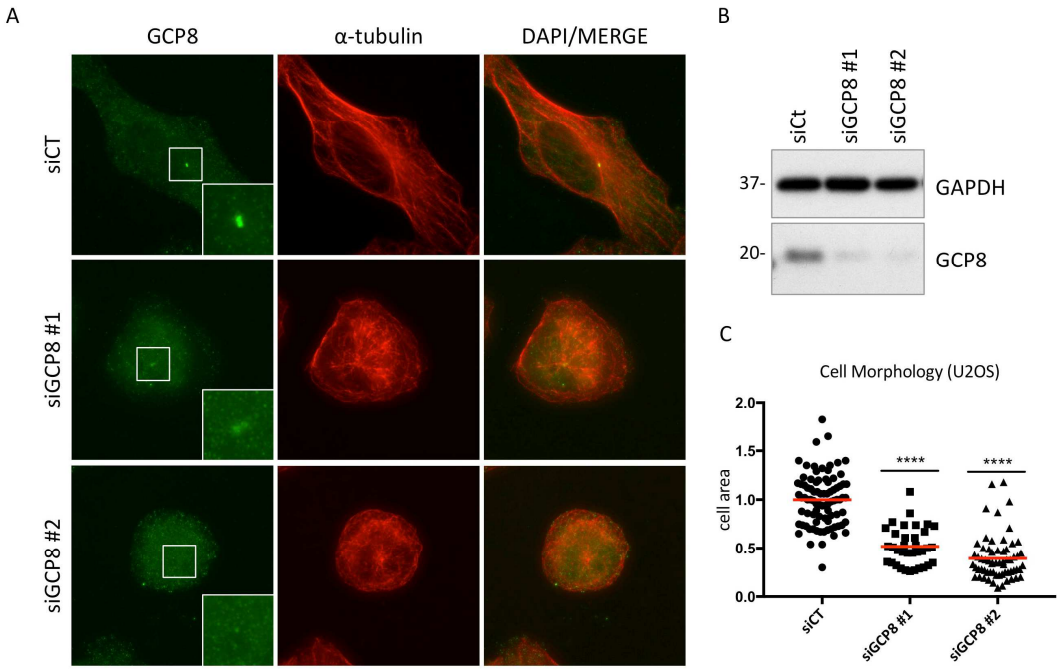


Figure 21. GCP8 depletion changes cell morphology. A) U2OS cells transfected with control or siRNAs against GCP8 were stained with GCP8 and α-tubulin antibodies. DNA was visualized with DAPI. B) After 72h whole cell lysates were analysed by WB with antibodies against the indicated proteins. C) The surface areas covered by cells were quantified, and individual values were plotted as normalized values relative to the control (n>30 cells from at least two independent experiments, ****p<0.0001).

2.1) GCP8 depletion does not compromise the actin cytoskeleton

In order to understand whether the effect on cell morphology after GCP8 depletion is caused by problems in actin organization I used phalloidin, which marks filamentous actin (F-actin), to analyze the actin cytoskeleton by IF. My result shows that the depletion of GCP8 does not have any obvious effect on the actin network, while it disorganizes the MT cytoskeleton (Figure 22), suggesting that the effect on the cell morphology may be MT dependent.

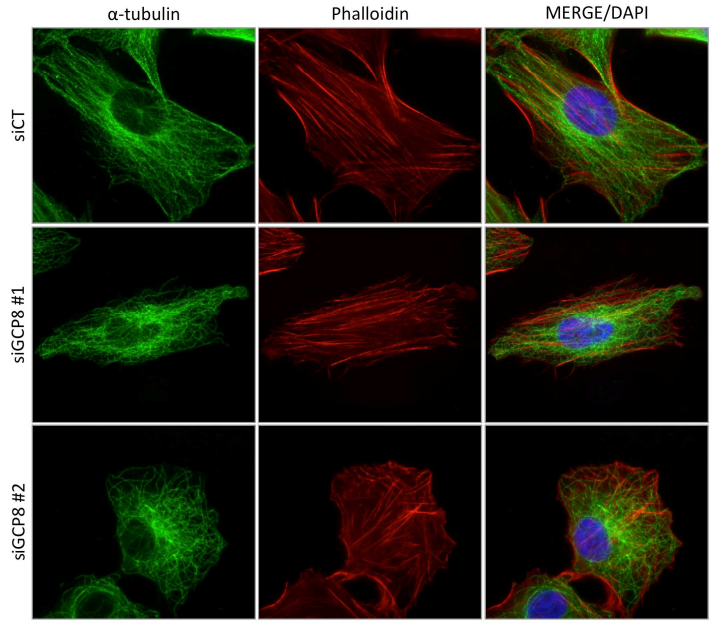


Figure 22. MT and actin organization after GCP8 depletion. RPE-1 cells were transfected with control and GCP8 siRNAs and stained with α-tubulin antibodies and phalloidin. DNA was visualized with DAPI

3) GCP8 depletion affects MT regrowth

To better understand the relevance of GCP8 in the regulation of MT nucleation during interphase I decided to re-evaluate this issue in RPE-1 cells. In our original published characterization of GCP8 in U2OS cells we found that GCP8 was required for robust MT regrowth at centrosomes, at least in interphase cells. However, I found that in RPE-1 cells, under conditions where the centrosomal levels of γTuRC proteins are similar to the control cells, MT regrowth at the centrosome was stimulated (Figure 23A). Curiously, instead of affecting MT nucleation in a similar way, a second oligo showed the opposite effect, reducing both γ-tubulin and nucleation at the centrosome (Figure 23B-D). We observed that the target sequence for oligo 1 is in a region with a proposed splice site, while the oligo 2 binds to a region within the 3´UTR non-coding sequence (Figure 23F). To test experimentally that differential targeting of potential GCP8 splice variants may explain the puzzling results, I knocked down GCP8 with both oligos in different cell lines and analysed the results by WB. Interestingly, differences in the band pattern can be appreciated between both oligos and in different cell lines, suggesting the existence of several GCP8

isoforms (Figure 23E). Overall, this result suggests that the differences observed in the MT nucleation capacity may be explained by differences in the efficiency of depletion of various GCP8 variants.

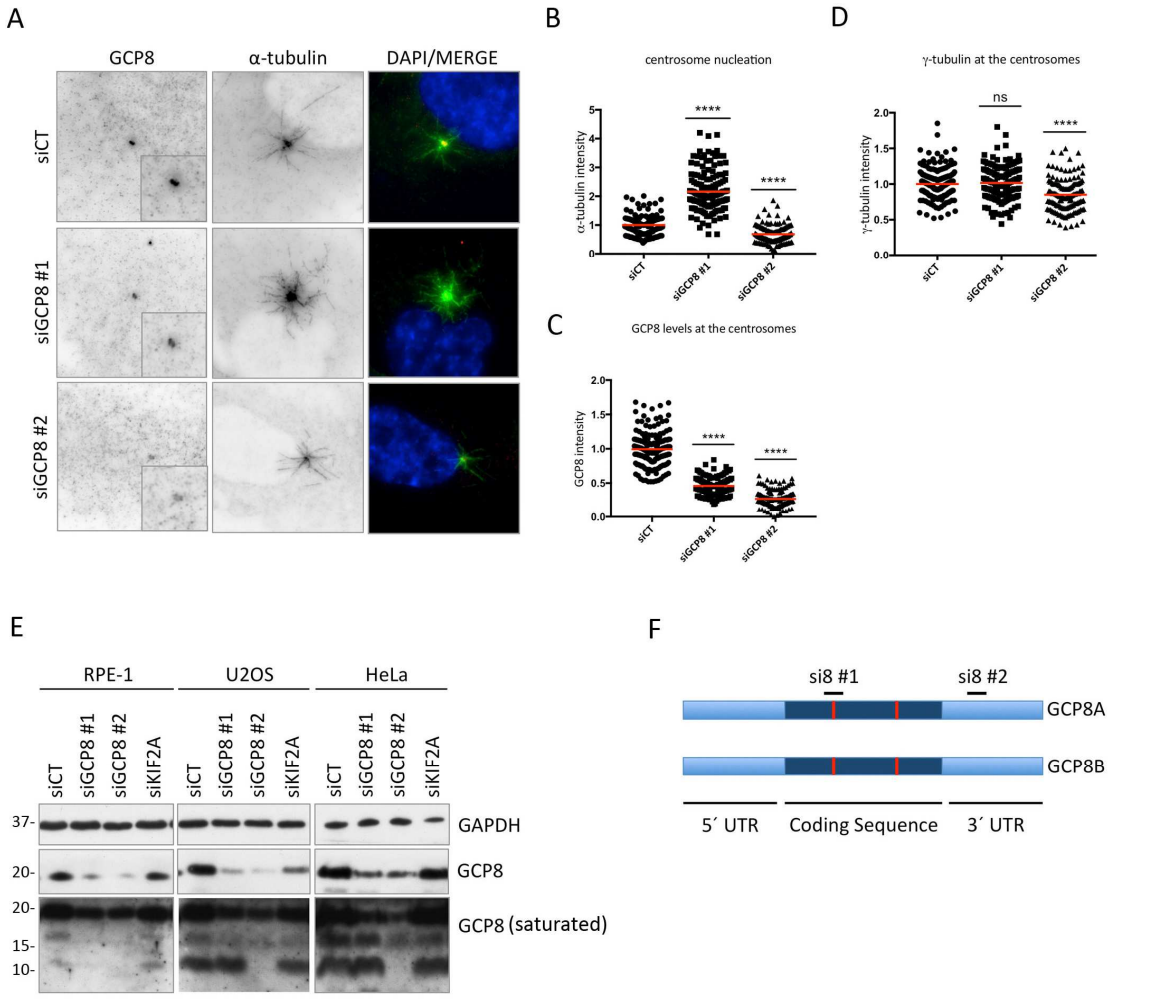


Figure 23. GCP8 depletion affects centrosome nucleation. A) RPE-1 cells were transfected with control or GCP8 siRNAs and were subjected to a MT regrowth assay. MTs were depolymerized on ice for 30 min and MT were allowed to regrow by warming (time point 10s) before fixation and staining with GCP8 and α-tubulin antibodies. B) The intensities of MT asters were quantified, and individual values were plotted as normalized values relative to the control (n>120 cells from 5 independent experiments, ****p<0.0001). C-D) GCP8 and γ-tubulin intensity around the centrosome were quantified and individual values were plotted as normalized values relative to the control (n>140 cells from 5 independent experiments, ns p>0.05 ns p>0.05, ****p<0.0001) E) Immunoblot for GAPDH and GCP8 from soluble fraction from RPE-1, U2OS and HeLa cells after control and GCP8 siRNAs transfection. F) Schematic representation of GCP8A/B mRNA and the siRNAs target regions. Splice sites are shown as red vertical lines.

4) Identification of new GCP8 interactors

Usually, protein function can be only understood in the context of interaction networks. GCP8, as part of the γTuRC, localizes at the centrosome and previously used techniques to identify interactors have limitations due to the largely insoluble nature of this organelle. Moreover, unpublished data from our laboratory suggested a potential role of GCP8 in the regulation of the actomyosin network, components of which also associate with the insoluble cellular fraction. Therefore, additional approaches were required to overcome this limitation.

In the last years, proximity-dependent biotin identification (BioID) has been established as a powerful technique to identified physiologically relevant protein interactions in living cells. This method takes advantage of a biotin protein ligase of Escherichia coli (BirA) to biotinylate proteins in a proximity-dependent manner. The biotin ligase is fused to a protein of interest, and then introduced into cells where it will biotinylate vicinal proteins upon supplementation of the culture medium with biotin. The biotinylated proteins are affinity-purified and finally identified by mass spectrometry. With this approach I aimed to identify proteins that are in proximity to GCP8 in its natural cellular environment (Figure 24).

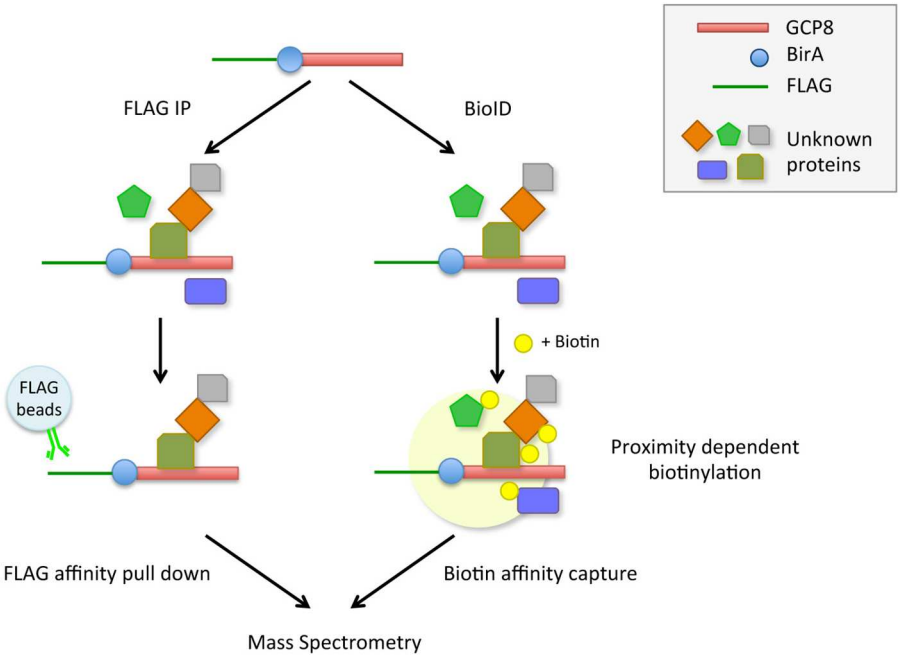


Figure 24. Schematic model of BioID and FLAG pull down methods. Expression of a FLAG-BirA-GCP8 protein allow us to purify GCP8 by two independent techniques. In the FLAG pull down, agarose beads coupled to anti-FLAG antibody bind to the FLAG-tagged GCP8 construct. FLAG elution leads to the detection of GCP8 interacting proteins with high specificity. In case of the BioID, BirA-tagged GCP8 leads to the selective biotinylation of proteins proximal to that fusion protein. Biotinylated proteins can be purified with streptavidin-coupled beads.

To perform BioID with GCP8 as bait I created stable cell lines expressing FLAG-BirA-GCP8 by co-transfecting HeLa flp-in cells (these cells contain a single stably integrated FRT site at a transcriptionally active genomic locus) with a GCP8-FLAG-BirA plasmid and a pOG44 vector, which expresses the Flp-recombinase. This enzyme allows the integration of the expression vector to the same locus in every cell, ensuring homogeneous levels of gene expression.

Since this technique identifies proteins based on proximity and since this may depend on the position of the tag, I also generated a stable cell line with the tag at the C-terminal part of GCP8 (GCP8-FLAG-BirA).

4.1) BirA-tagged GCP8 localizes and stimulates biotinylation at the centrosome

To test the feasibility of using BioID with GCP8 as bait I analyzed by IF the localization and biotinylation capacity of the GCP8 fusions with BirA. Similar to endogenous GCP8 the fusion proteins localized to the centrosomes and bind to the γTuRC (Figure 25A-B).

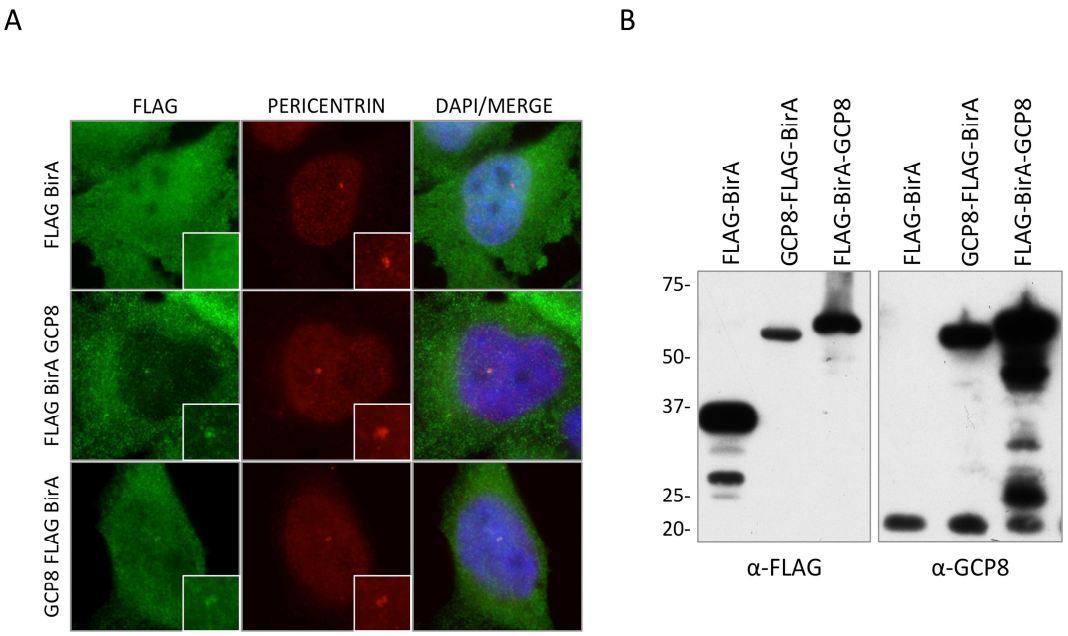


Figure 25. GCP8 fusions with BirA localize at the centrosome. A) HeLa cells expressing FLAG-BirA alone or fused to GCP8 were stained with FLAG and pericentrin antibodies. DNA was visualized with DAPI. B) Immunoblot detecting FLAG and GCP8 in HeLa cells stably expressing FLAG-BirA, or BirA fused to GCP8.

Moreover, detection of intracellular biotinylated proteins using Alexa Fluor594 streptavidin specifically labeled centrosomes in cells expressing the GCP8 fusions with BirA but not in cells expressing BirA alone (Figure 25A-B). Together these results suggest that I successfully generated tools that allow applying the BioID approach to detect novel proximity interactors of GCP8.

In addition, taking advantage of the presence of a FLAG tag in the fusion proteins, I also performed standard FLAG IPs to complement the BioID data.

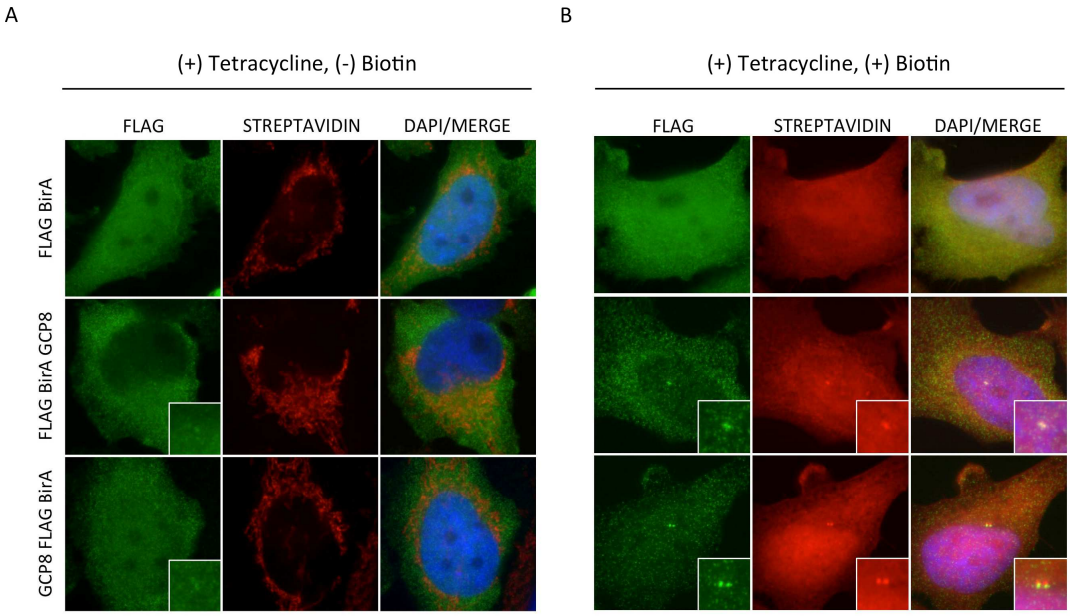


Figure 26. GCP8 fused to BirA stimulates biotinylation at the centrosome. A) Uninduced HeLa FLAG-BirA or fusions of GCP8 and BirA were fixed in methanol after 24h incubation with biotin and stained against FLAG antibody and streptavidin-Alexa Fluor 568. B) Tetracycline induces HeLa FLAG-BirA or FLAG-BirA-GCP8 constructs were fixed in methanol after 24h incubation with tetracycline plus biotin and stained against FLAG antibody and streptavidin-Alexa Fluor 568

4.2) Classification of the purified protein candidates

In collaboration with the laboratory of Brian Raught at the University of Toronto, the tagged GCP8 constructs were affinity-purified and associated proteins were analyzed by mass spectrometry (Figure 24). In order to classify the mass spectrometry results, high confidence interactors were defined as those with a SAINT score equal to 1. Thus, I finally got 310 protein candidates for the FLAG pull down and 209 for the BioID. Moreover, in spite of getting some differences between the N and the C-terminal construct, they show a high degree of similarity (Figure 27A and B). However, stronger differences can be seen if comparing the whole protein lists between the FLAG pull down and the BioID (Figure 27C).

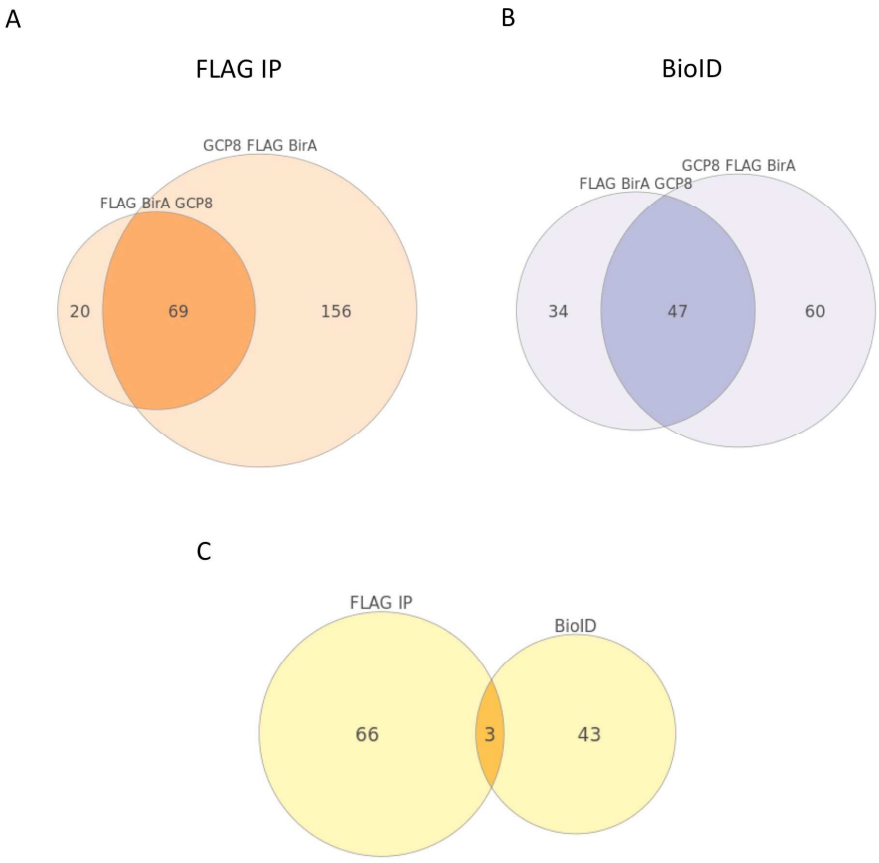


Figure 27. Diagram depicting the results of the BioID and FLAG pull-down experiments. A-B) FLAG pull-down and BioID protein candidates were compared for the two different bait proteins, BirA-FLAG-GCP8 and GCP8-FLAG-BirA. Numbers in circles refer to the number of proteins significantly enriched relative to the control for each of the baits. The overlapping areas represent proteins identified in both samples. C) The combined protein candidates identified with both baits were compared between the FLAG pull-down and the BioID. Numbers in circles refer to the total number of identified proteins significantly enriched relative to the controls for each of the two methods. The overlapping areas represent proteins identified in with both methods.

As expected, GCP2, which is the binding partner of GCP8, was the top hit in both cases, confirming the feasibility of the obtained results (Figure 28A and C). Curiously, while essentially all the γTuRC subunits were identified in the FLAG pull down (Figure 28A), GCP5 and MZT1 were not detected in the BioID (Figure 28C), suggesting that these proteins are not in close proximity to GCP8.

Interestingly, we identified KIF2A (Figure 28A), a member of the kinesin-13 protein family, in the FLAG pull-down and in an independent GCP3 pull-down assay performed with primary cortical neurons (Lüders group, unpublished). Since another member of this

protein family was previously reported to negatively regulate MT nucleation in vitro (Wieczorek M, 2015), I decided to study whether KIF2A may regulate MT nucleation by γTuRC during interphase in culture cells.

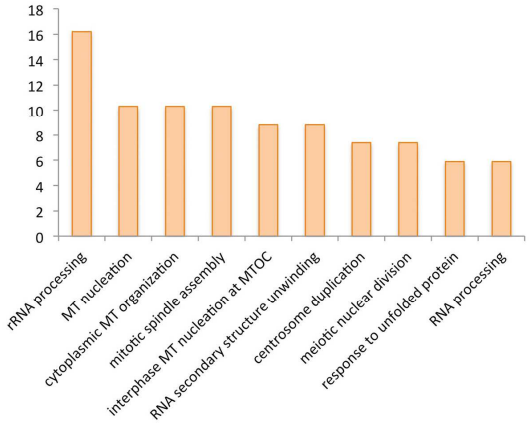
To learn more about the protein candidates, I performed a gene ontology (GO) analysis according to the GO term "biological process", using the database for annotation visualization and integrated discovery (DAVID) online software (Figure 28B and D). As expected, several categories such as "MT nucleation" or "cytoplasmic MT organization" were significantly enriched in both the BioID and the FLAG pull down. The biological significance of the other categories, despite suggesting a potential role of GCP8 in other cellular functions, has not been analyzed during my thesis.

A

FLAG IP

FLAG BirA GCP8			GCP8 FLAG BirA		
Pos.	Gene	Total	Pos.	Gene	Total
1	**GCP2**	4563	1	**GCP2**	3329
2	**GCP3**	2881	2	**GCP3**	2085
3	**TUBG1**	2809	3	**TUBG1**	2052
	...			...	
5	**GCP4**	956	8	**GCP4**	510
6	**GCP6**	911	9	**GCP6**	441
7	**GCP5**	531		...	
	...		11	**GCP5**	336
15	**MZT1**	230		...	
	...		20	**MZT1**	171
65	**KIF2A**	32		...	
			99	**KIF2A**	78
				...	
			202	**NEDD1**	31

B



C

BioID

FLAG BirA GCP8			GCP8 FLAG BirA		
Pos.	Gene	Total	Pos.	Gene	Total
1	**GCP2**	3551	1	**GCP2**	1948
2	**NEDD1**	920	2	**NEDD1**	789
3	**GCP3**	766	3	**GCP3**	934
	...			...	
48	**GCP4**	28	67	**GCP6**	16
	...				
61	**GCP6**	19			

D

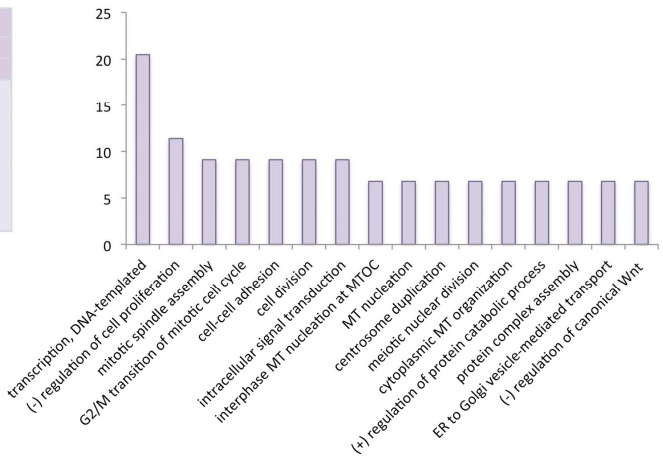


Figure 28. Classification of purified proteins. A-C) Schematic representation of the mass spectrometry data from the FLAG pull down and the BioID. Proteins were classified based on the total spectral counts compared to the control. B-D) The protein candidates from both approaches were submitted to a gene ontology analysis based on the GO term "biological process". The represented groups include those enriched categories ($p<0.01$) with at least a 5% of the genes from the total genes (involved genes/total genes).

5) GCP antibodies pull down KIF2A

KIF2A is a kinesin required for mitotic progression (*Ganem NJ & Campton DA, 2004*) and, as other kinesin-13 family members, was shown to have MT destabilizing activity (*Walczak et al., 2013*, *Miyamato T. et al., 2016*). KIF2A localizes at the centrosome but there are no published data describing interaction with the γTuRC. To validate KIF2A as γTuRC interactor, I immunoprecipitated γTuRC using antibodies against GCP8, GCP2, or GCP3. The results show that KIF2A co-immunoprecipitated in all cases (Figure 29).

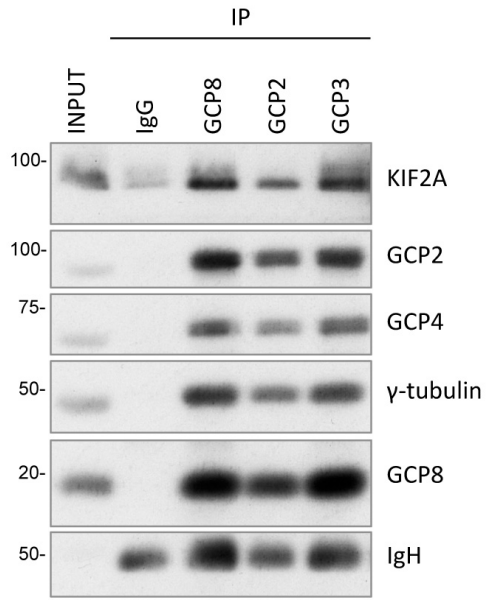


Figure 29. GCP8 pulls down KIF2A. Immunoblot for KIF2A and various γTuRC subunits as indicated of samples immunoprecipitated with GCP8, GCP2 or GCP3 antibodies.

5.1) GCP8 pulls down KIF2A through its C-terminus

To characterize this interaction in more detail, I transfected HeLa cells with different GCP8 fragments fused to a GFP-tag. Then, I pulled-down the GFP-tag and analysed the differences in the binding capacity between constructs by WB. I found that the GCP8 C-terminus, but not the N-terminus, interacts with endogenous KIF2A (Figure 30). In contrast,

endogenous γTuRC subunits were specifically associated with the N-terminal region of GCP8.

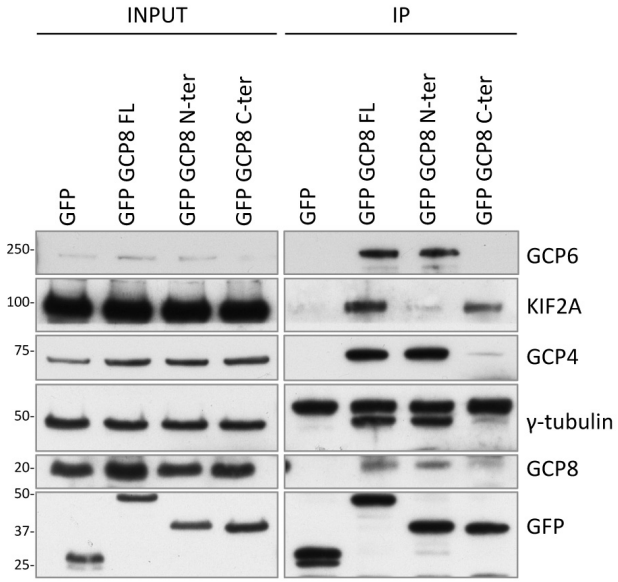


Figure 30. Characterization of GCP8-KIF2A binding region. Immunoblot for KIF2A, γTuRC subunits and GFP from inputs and immunoprecipitated lysates as indicated

5.2) GCP8 regulates the levels of KIF2A at the centrosomes

KIF2A was known to localize at the centrosome and I speculated that at least some of the centrosomal KIF2A may depend on interaction with GCP8. To address this question, I quantified the levels of KIF2A at the centrosome in RPE-1 cells after GCP8 depletion. Indeed, my results suggest that in absence of GCP8 the levels of KIF2A at the centrosomes are significantly reduced (Figure 31A and 4B).

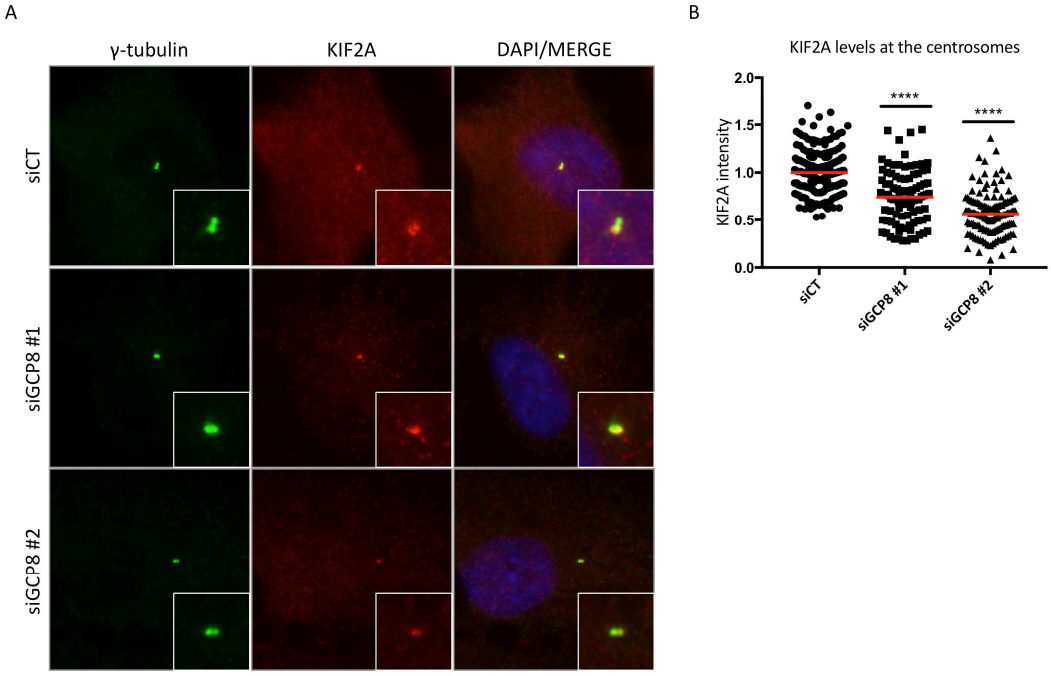


Figure 31. Effect of GCP8 depletion on KIF2A centrosomal levels. A**)** Interphase RPE-1 cells transfected with either control RNA or siRNA against GCP8 were stained with γ-tubulin and KIF2A antibodies. DNA was labelled with DAPI. The insets show magnifications of centrosomal areas. B) KIF2A fluorescence intensity at the centrosome was quantified and individual values were plotted as normalized values relative to the control (n>90 cells from four independent experiments, ****p<0.0001).

5.3) GCP8 partially co-localizes with KIF2A

Previous work localized KIF2A at the subdistal appendages and the proximal ends of both centrioles (Tatsuo Miyamoto K. H., 2015) ,whereas we expected GCP8 to be associated with the PCM, similar to other γTuRC subunits (Neus Teixidó-Travesa, 2010) (Figure 32A). To analyze in detail the localization of both proteins at the centrosome I stained RPE-1 cells with antibodies against GCP8, Cep170, as a marker for subdistal appendages and the centriole proximal end (similar to the described localization of KIF2A), and centrin, a marker for the distal end of centrioles (Gregory Mazo, 2016). My results indicate that GCP8 and KIF2A partially co-localize at the proximal end of the centrioles (Figure 32B).

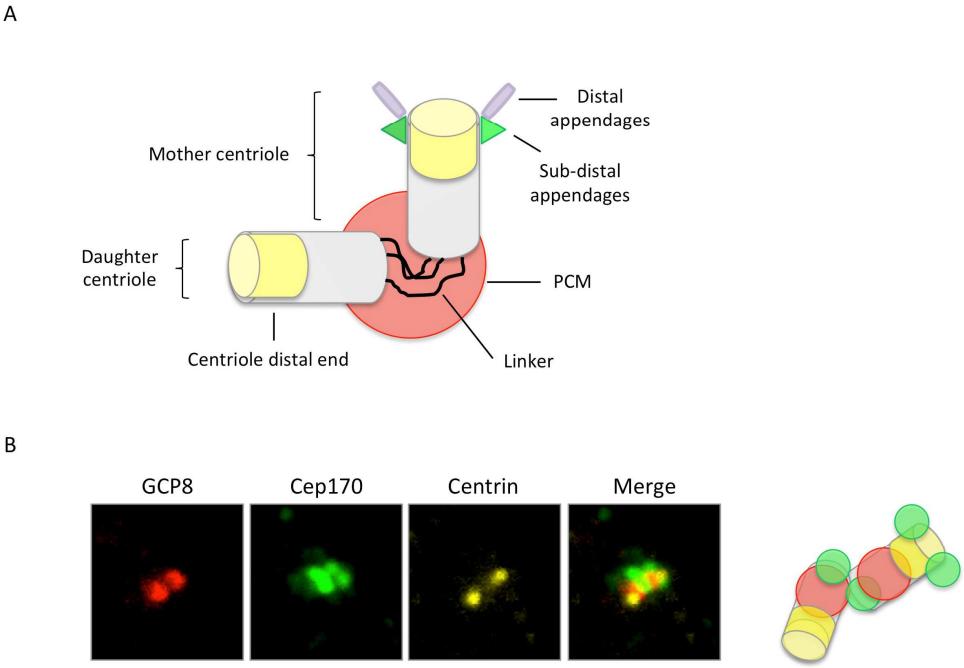


Figure 32. GCP8 and KIF2A centrosome localization. A) Schematic model of a centrosome. B) RPE-1 cells were fixed and stained with antibodies against GCP8, CEP170 and centrin. Schematic model of the centrosomal markers disposition.

5.4) KIF2A depletion stimulates MT regrowth

I have previously shown that GCP8 depletion increases MT nucleation under conditions where the γTuRC levels are not affected (Figure 33A). Since KIF2A is known to have MT-depolymerizing activity, we speculated that it may negatively regulate MT regrowth. Therefore, I decided to knockdown KIF2A by using siRNA and analyse the effect on MT regrowth (Figure 33A and B). Indeed, the depletion of KIF2A dramatically increased MT regrowth at the centrosome (Figure 33C), despite the fact that the γ-tubulin levels at the centrosome were even slightly lower than in controls (Figure 33D).

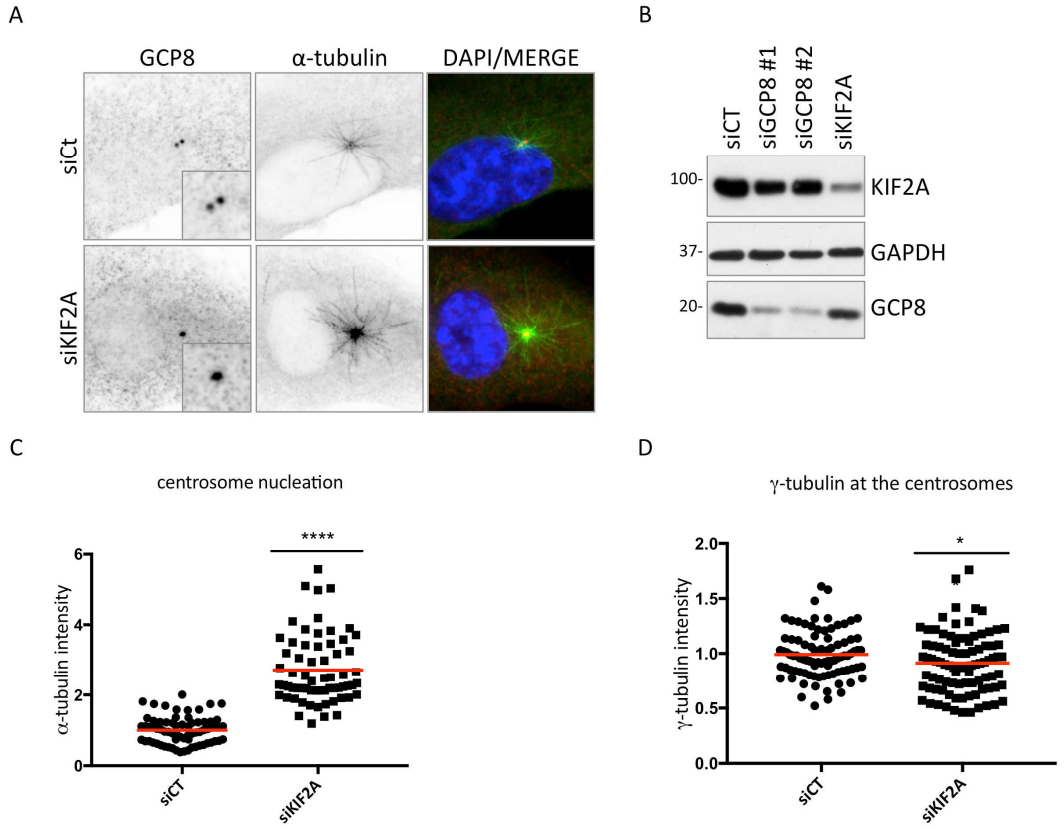


Figure 33. Effect of KIF2A depletion on centrosomal regrowth. A) RPE-1 cells were transfected with control or KIF2A siRNA and were subjected to a MT regrowth assay. MTs were depolymerized on ice for 30min and after warming MTs were allowed to regrow (time point 10s) before fixation and staining with antibodies against GCP8 and α-tubulin. The insets show magnifications of centrosomal areas. B) Immunoblot for KIF2A, GAPDH and GCP8 from RPE-1 cell extract after control, GCP8 and KIF2A siRNAs transfection. C) The intensities of MT asters were quantified, and individual values were plotted as normalized values relative to the control (n>60 cells from three different experiments, ****p<0.0001). D) γ-tubulin intensity at the centrosome was quantified and individual values were plotted as normalized values relative to the control (n>90 cells from three independent experiments, *p<0.05)

5.5) Expression of the N-terminal or C-terminal fragment of GCP8 does not affect KIF2A levels at the centrosome

At this point, I have shown that the depletion of GCP8 reduces the levels of KIF2A at the centrosome and that GCP8 binds KIF2A through the C-terminal region. So, I was wondering whether the overexpression of any of the two GCP8 fragments may be used as dominant-negative mutant, to reduce the levels of KIF2A at the centrosome. I transfected cells and after expression of each fragment for 48 hours I fixed the cells and analyzed the centrosomal levels of KIF2A by IF. Unfortunately, none of the fragments significantly changed the levels of KIF2A at the centrosome when overexpressed (Figure 34A-B).

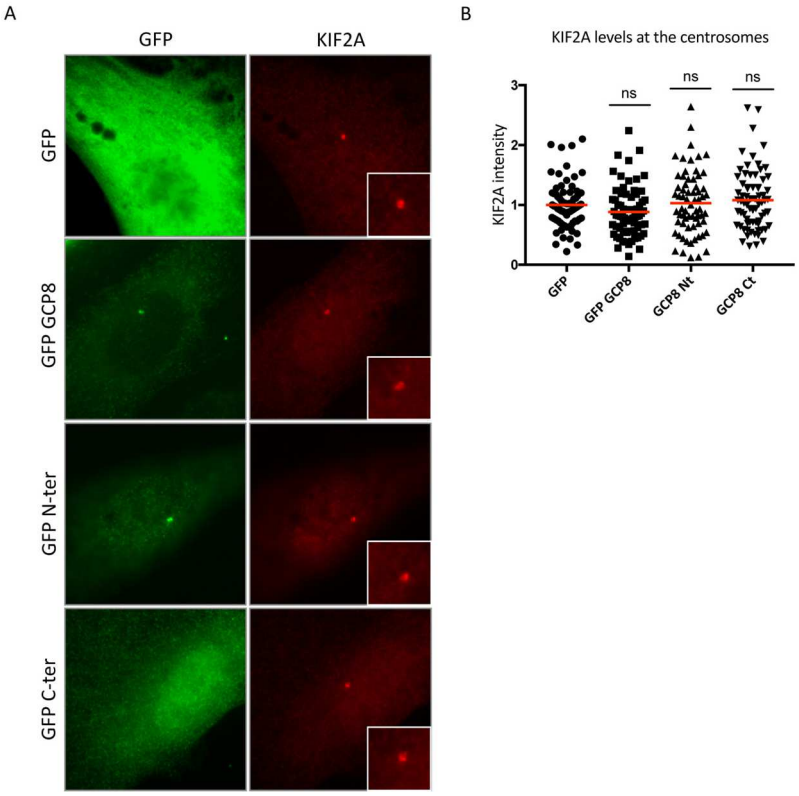


Figure 34. Effect on KIF2A localization in cells expressing GCP8 fragments. A) RPE-1 cells were transfected with different GCP8 constructs during 48h and stained with antibodies against GFP and KIF2A. The insets show magnifications of centrosomal areas. B) The intensities of KIF2A signal at the centrosome was quantified and individual values were plotted as normalized values relative to the control (n>60 cells from three independent experiments, ns p>0.05).

6) The overexpression of untagged GCP8 does not affect MT regrowth

As shown above, the overexpression of GFP-tagged GCP8 did not increase the levels of KIF2A at the centrosome. However, it may be that the GFP-tag affects the functionally of GCP8. I addressed this issue by repeating the experiment with an untagged GCP8 construct. However, since GCP8 and KIF2A antibodies were raised in the same species (rabbit), I analyzed the effect of this new construct in a complementary assay. Considering that the depletion of GCP8 or KIF2A stimulates MT regrowth at the centrosome, I tested whether the overexpression of GCP8 had any effect in this assay. However, neither GFP alone as control nor the untagged GCP8 construct affected MT regrowth at the centrosome (Figure 35A-D).

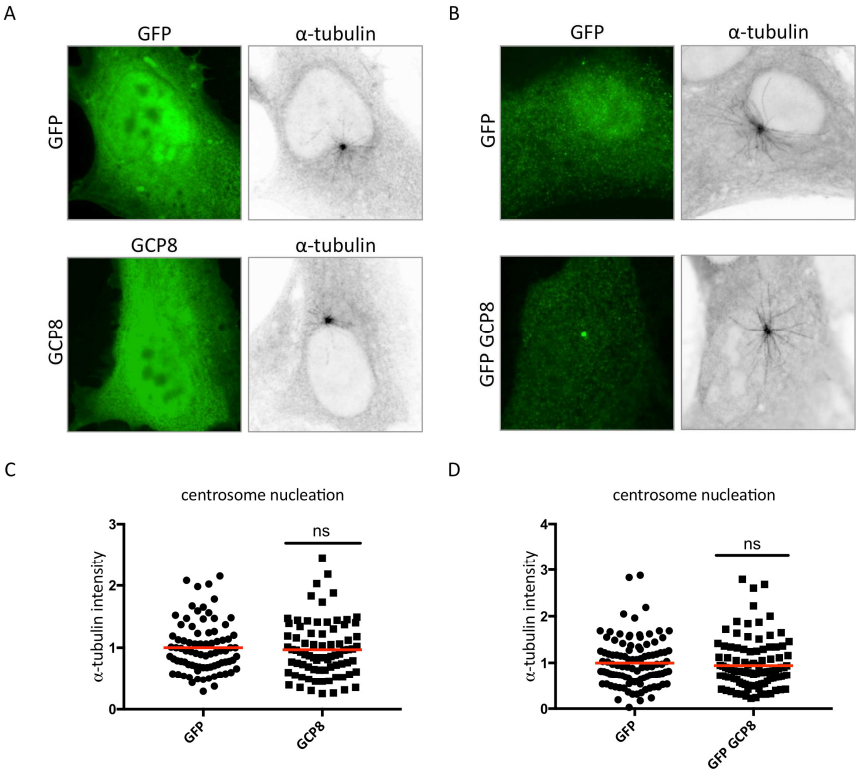


Figure 35. Effect of GCP8 expression on centrosomal MT regrowth. A-B) RPE-1 cells expressing GFP, GFP-GCP8 or untagged GCP8 were subjected to a MT regrowth assay. MTs were depolymerized on ice for 30 min and after warming MTs were allowed to regrow (time point 10 s) before fixation and staining with antibodies against GFP, GCP8 and α-tubulin. C-D) The intensities of MT asters were quantified, and individual values were plotted as normalized values relative to the control (n>100 cells from at least three different experiments, ns p>0.05)

7) Expression of GCP2Δ24 does not affect the levels of KIF2A at the centrosome

The data presented so far suggested that KIF2A interacts with γTuRC through GCP8, to negatively regulate nucleation at the centrosome. To demonstrate this more directly I considered a strategy that I had used in another similar project. Similar to GCP8, the small γTuRC subunit MZT1 also mediates recruitment of γTuRC regulators, in this case activators and targeting factors. Moreover, in analogy to the binding of GCP8 to the N-terminus of GCP2, MZT1 binds to the N-terminus of GCP3. To test the effects of specifically disrupting the interaction of MZT1 with GCP3 I had performed rescue experiments by depleting endogenous GCP3 and expressing an RNAi-resistant MZT1-binding mutant of GCP3. As expected, under GCP3 depletion conditions, the levels of centrosomal γ-tubulin were reduced and the mitotic index increased due to spindle defects, and the recombinant wild-

type GCP3 rescued these phenotypes (Figure 36A-C). However, these defects were not rescued by the MZT1-binding mutant of GCP3. Moreover, in the absence of MZT1-binding, the recruitment of the γTuRC-activating CM1 domain was reduced (Figure 36C). Having demonstrated the viability of such an approach I devised a similar strategy for GCP8.

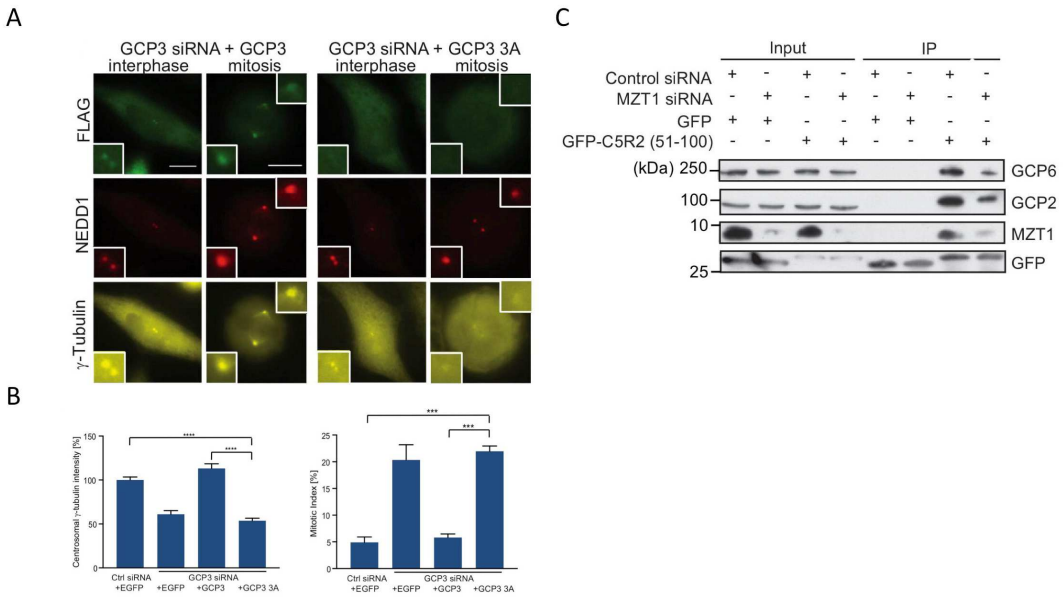


Figure 36. MZT1 phenotypes. A) Immunofluorescence images of HeLa cells depleted of GCP3 and expressing RNAi-resistant FLAG-tagged wild-type or 3A mutant GCP3. Cells were stained with anti-FLAG, anti-NEDD1 and anti-γ-tubulin antibodies. Scale bar: 10 µm. (B) Centrosomal γ-tubulin staining was quantified in interphase HeLa cells transfected with control or GCP3 siRNA, and with plasmid expressing FLA–EGFP, and FLAG–GCP3 or FLAG–GCP3 3A mutant. Mean intensities were plotted relative to control cells (set to 100%); *n*=60 centrosomes per condition combined from three independent experiments. (C) The mitotic index was scored in cells transfected with control or GCP3 siRNA, and with plasmid expressing FLAG–EGFP, and FLAG–GCP3 or FLAG-GCP3 3A mutant; *n*=3 experiments, >200 cells per condition in each experiment. All quantitative results are mean ±s.e.m. ***p<0.001; ****p<0.0001 (unpaired *t*-test compared with wild type or as indicated). D) HEK293 cells transfected as in A were subjected to immunoprecipitation (IP) with anti-GFP antibodies. Samples were analysed by western blotting with antibodies against the indicated proteins.

I have previously shown that the overexpression of a GCP2 construct that lacks the first 24 amino acids significantly reduces the levels of GCP8 at the centrosome. Hence, unlike the previous situation, the overexpression of GCP2Δ24 may be sufficient to reproduce the GCP8 depleted phenotypes. Moreover, the depletion of KIF2A stimulates MT regrowth at the centrosome. Hence, in order to understand whether GCP8 negatively regulates MT

regrowth by impairing the binding of KIF2A to the γTuRC, I first analyzed the levels of KIF2A at the centrosome after overexpressing the GCP2Δ24 construct. Unfortunately, similar to the effect that I have described after overexpressing GCP8, the overexpression of this GCP2 mutant construct does not reduce the levels of KIF2A at the centrosome (Figure 37A and B). This result may suggest that the remaining GCP8 at the centrosome is sufficient to keep the normal levels of KIF2A at this structure.

On the other hand, in order to evaluate the functionality of these constructs I performed a regrowth assay after overexpressing both the GCP2 wild type and the mutant. Unexpectedly, in both cases centrosomal regrowth was reduced (Figure 37C), something that was particularly strange in the case of the GCP2 wild type construct. This result prompt us to consider that these constructs are not functional.

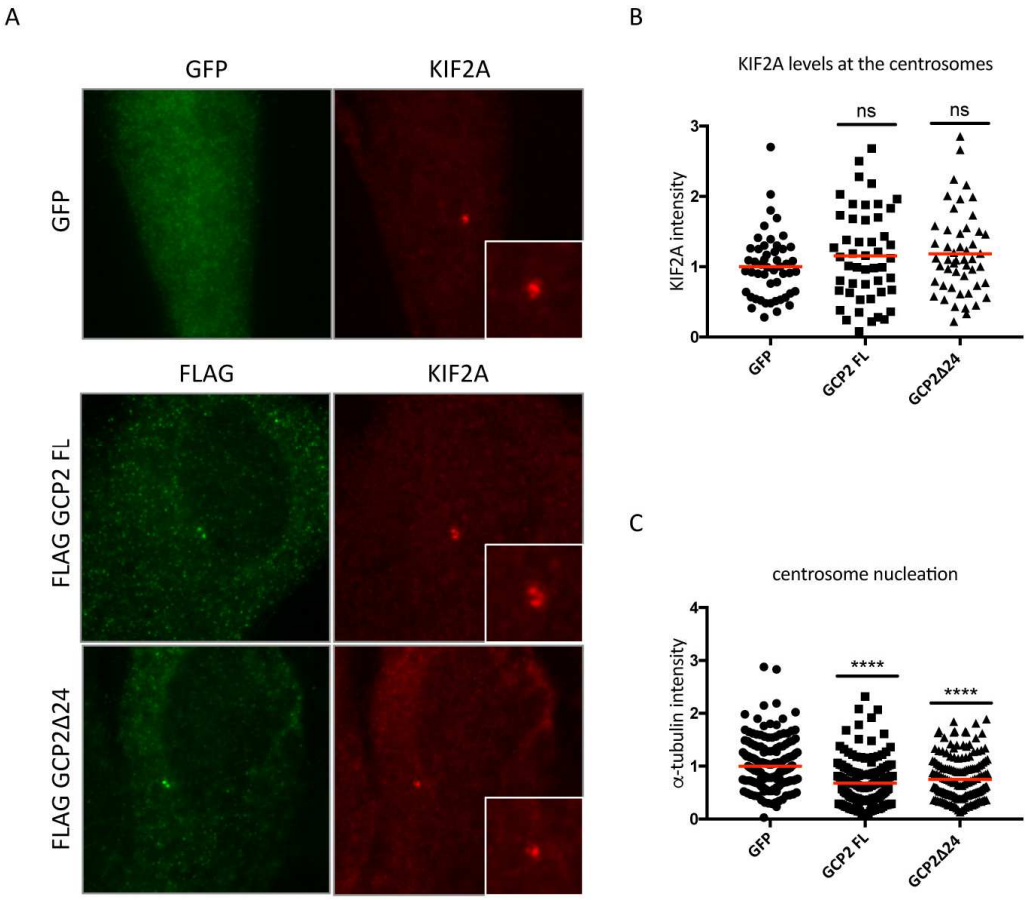


Figure 37. Effect of GCP2Δ24 on centrosome regrowth and KIF2A levels. A) RPE-1 cells were transfected with GFP, FLAG-GCP2Δ24 or FLAG-GCP2 FL and stained for KIF2A, GFP and FLAG antibodies. The insets show magnifications of centrosomal areas. B) KIF2A intensity at the centrosome was quantified and individual values were plotted as normalized values relative to the control (n>50 cells from two independent experiments, ns p>0.05) C) These cells were also subjected to a MT regrowth assay. MTs were depolymerized on ice for 30min and after warming up MT were allow to regrow (time point 10s) before fixation and staining against GFP, and α-tubulin antibodies. The intensities of MT asters were quantified, and individual values were plotted as normalized values relative to the control (n>150 cells from five independent experiments, ****p<0.0001, ns p>0.05)

8) GCP8 and KIF2A regulate Golgi nucleation

MT nucleation can take place at different MTOCs, including the Golgi apparatus. Curiously, although it was not a top hit, the peripheral membrane component of the cis-Golgi GOLGA2/GM130 was one of the few candidates present in both the BioID and the FLAG pull down. GM130 is essential for recruiting MT nucleation factors to this organelle (Sabrina Rivero, 2009).

I decided to evaluate whether GCP8 and KIF2A were implicated in nucleation from the Golgi. To address this question, I treated RPE-1 cells with nocodazole to depolymerize the

MTs and to disperse the Golgi in individual vesicles throughout the cytoplasm. After nocodazole washout the cells were incubated on ice to completely remove the remaining MTs and MT growth can be visualized from both the centrosome and the individual Golgi vesicles. Interestingly, similar to regrowth from the centrosome, I observed an increase in the regrowth capacity both after GCP8 or KIF2A depletion (Figure 38A and D).

An interesting feature common to GCP8 or KIF2A depletion was that the morphology of the Golgi was altered. I observed clustering of the Golgi membranes around the centrosome that resulted in the loss of the typical ribbon organization and could be measured by a reduction in the Golgi-occupied area (Figure 38C). Moreover, the intensity of GM130 staining was increased (Figure 38B). Note that in contrast to KIF2A or GCP8 depletion with siRNA #1, siRNA#2 did not reduce the average area occupied by the Golgi, which may be explained by the reduction in centrosomal nucleation in these cells. Overall, these results suggest that increasing the nucleation activities at the centrosome and the Golgi disrupts the higher order ribbon structure of the Golgi and causes clustering of Golgi membranes around the centrosome.

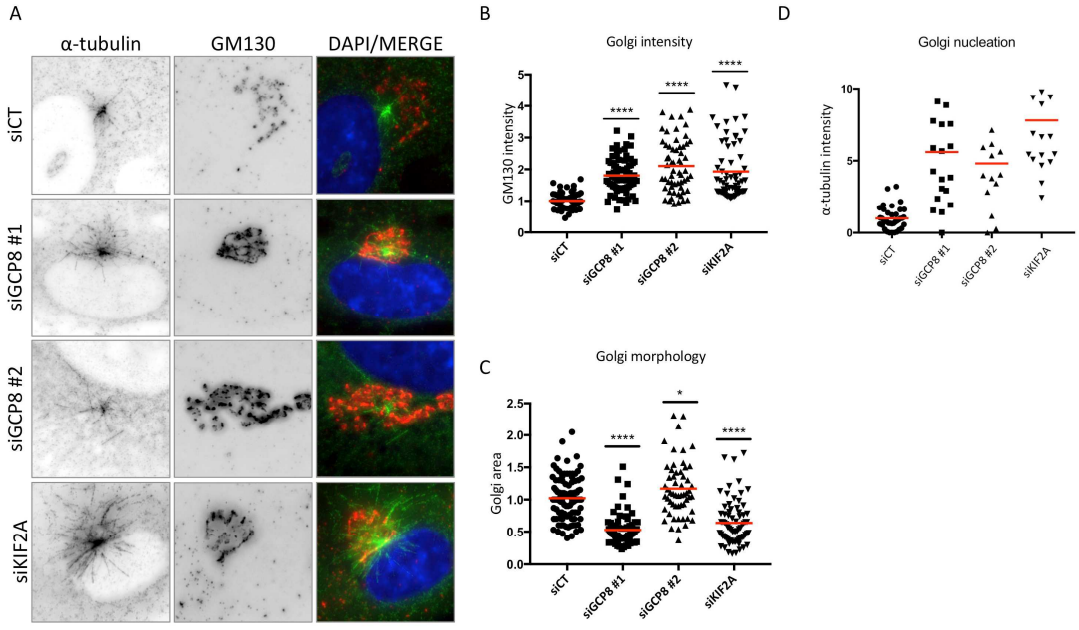


Figure 38. GCP8 or KIF2A depletion stimulates nucleation from the Golgi. A) RPE-1 cells were transfected with control or KIF2A siRNA and were subjected to a MT regrowth assay. MTs were depolymerized on ice for 30 min and after warming MTs were allowed to regrow (time point 10 s) before fixation and staining with antibodies against GM130 and α-tubulin. B) GM130 intensity was quantified and individual values were

plotted as normalized values relative to the control (n>60 cell from three independent experiments, ****p<0.0001). C) Golgi morphology was measured by quantifying the area inside the GM130 perimeter (n>50 cell from three independent experiments, ****p<0.0001, *p<0.05). D) The intensity of tubulin signal coming from the Golgi was quantified by selecting the Golgi area after removing the centrosome signal.

8.1) GCP8 or KIF2A depletion stimulate Golgi nucleating cells

Since γ-tubulin was shown to localize to the cis-Golgi in RPE-1 cells, I tested whether GCP8, apart from its know localization at the centrosome, also co-localizes with GM130 at the cis-Golgi. Despite the weak signal, a partial co-localization of GCP8 at the Golgi region could be detected, consistent with a role of GCP8 in the regulation of Golgi-associated γTuRC (Figure39).

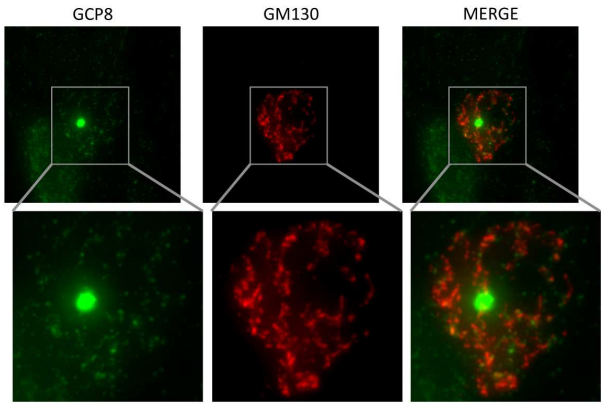


Figure 39. GCP8 and GM130 localization. RPE-1 cells were treated with 1% saponin buffer (to remove the soluble cell fraction before fixation and stained against GCP8 and GM130. The insets show magnifications of Golgi areas.

Since the tight clustering of the Golgi around the centrosome raised the concern that the close proximity of the two organelles may not allow accurate quantification of their nucleation activity, I performed a regrowth assay using nocodazole to disperse the Golgi vesicles throughout the cytoplasm. Under these conditions, cells with MTs growing from individual vesicles, well separated from centrosomes, can be counted. I observed, similar to the previous assay, GCP8 or KIF2A depletion increased the percentage of cells with MTs growing from the individual dispersed Golgi vesicles, (Figure 40A-B).

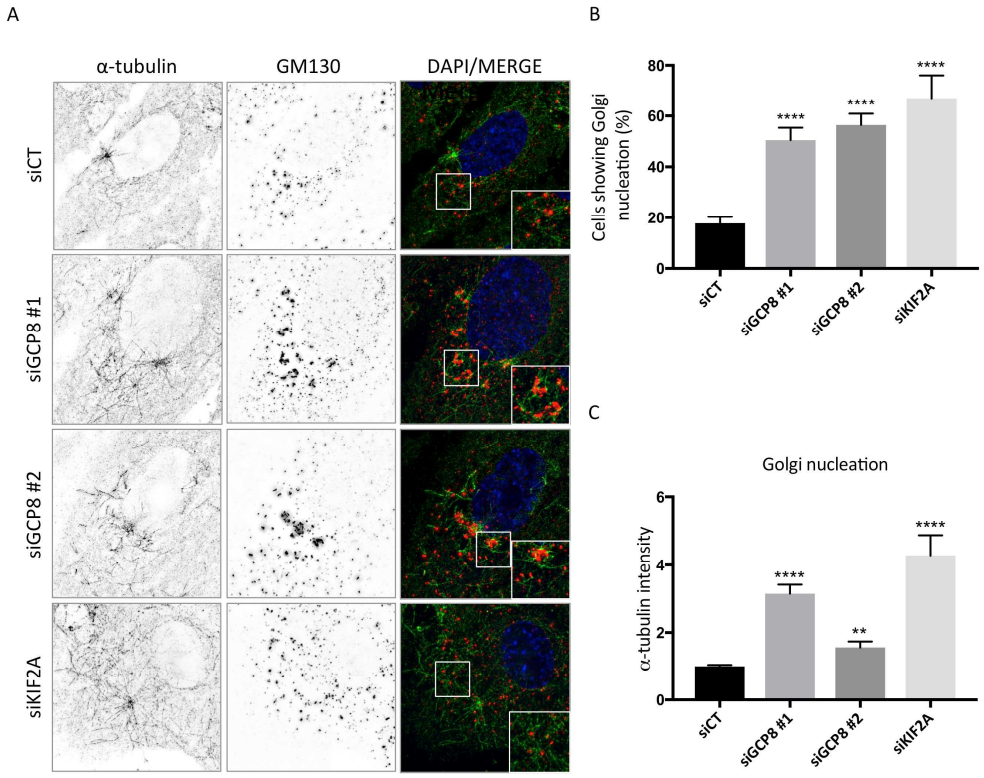


Figure 40. GCP8 or KIF2A depletion increases nucleation from dispersed Golgi vesicles. A) RPE-1 cells were transfected with control, GCP8 or KIF2A siRNAs and were subjected to a MT regrowth assay. MTs were depolymerized and the Golgi dispersed with 2,5µg/ml nocodazole during 3h at 37ºC followed by 30 min on ice. MTs were allowed to regrow by warming (time point 30 s) before fixation and staining against GM130 and α-tubulin. B) The number of cells with MTs growing from the Golgi were counted and plotted as percentages relative to the control (n=200 cells from at least two independent experiment ****p<0.0001). C) The intensity of tubulin signal coming from the Golgi was quantified by selecting the Golgi area after removing the centrosome signal (n>25 cells from at least two independent experiment ****p<0.0001, **p<0.01)

9) GCP8 stimulates centrosomal regrowth in a γTuRC dependent manner

Since the previous experiment in which I overexpressed the GCP2Δ24 construct did not clarify whether the effects after GCP8 depletion are γTuRC dependent or not I re-evaluate this issue by co-depleting GCP8 together with GCP2. So far, I analyzed the effect of the co-depletion in a regrowth assay. As expected, although the results are still preliminary, they suggest that the described effect on centrosomal regrowth essentially depends on the γTuRC (Figure 41A). Moreover, I also measured the area of the Golgi apparatus and,

according to the effect on the centrosomal regrowth, the co-depletion of GCP8 together with GCP2 recovers the normal Golgi area (Figure 41B).

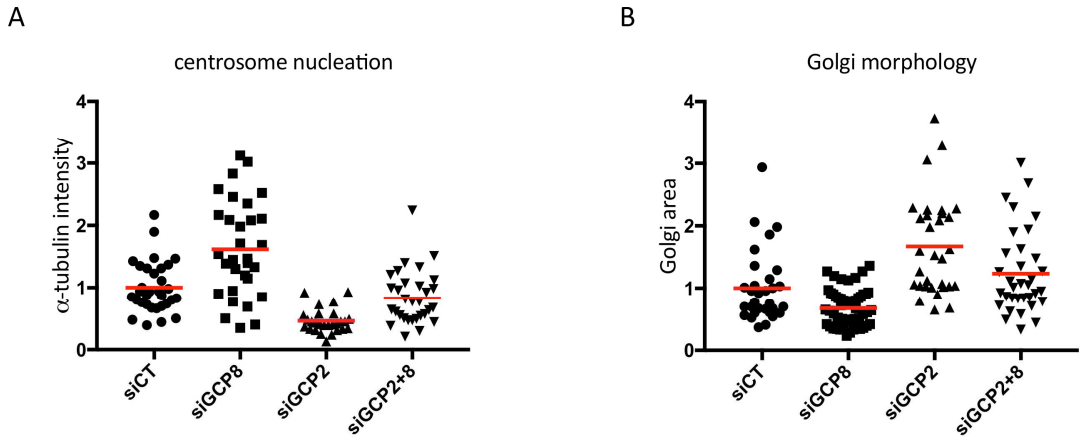


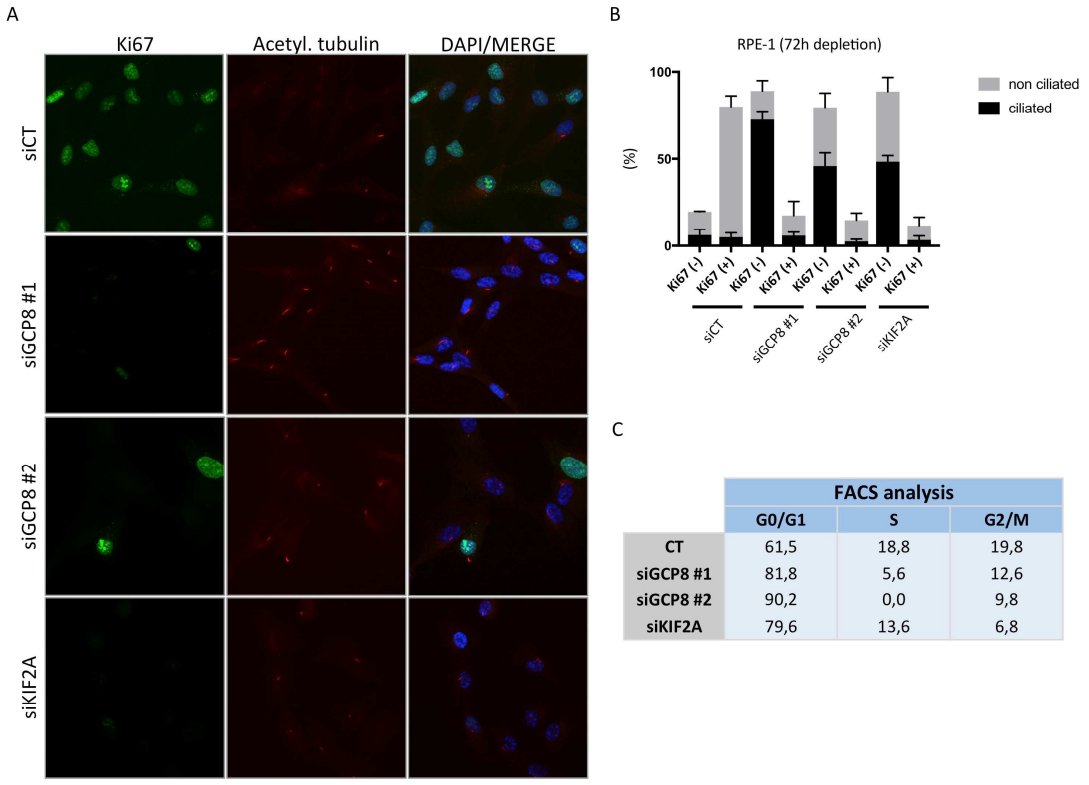
Figure 41. Effects of GCP8 and GCP2 co-depletion. A) The intensities of MT asters from a regrowth assay were quantified, and individual values were plotted as normalized values relative to the control (n>20 cells from one single experiment). B) Golgi morphology was measured by quantifying the area inside the GM130 perimeter (n>20 cells from one single experiment).

10) GCP8 and KIF2A regulate cell cycle progression

During my studies I noticed that RPE-1 cells depleted of GCP8 or KIF2A stopped proliferation. Since GCP8 is the only γTuRC subunit without a defined function during mitosis, I decided to evaluate whether GCP8 may regulate cell cycle progression in other ways.

A variety of cellular problems can trigger cell cycle arrest or exit in RPE-1 cells by different mechanisms. When cells are forced to exit the cell cycle, for example by serum starvation, they typically assemble a primary cilium.

I directly evaluated the effect on cell cycle progression after 72 hours of depletion in complete media by using the cell proliferation marker Ki67. Interestingly, in absence of GCP8 or KIF2A the number of cells arrested in G0 phase (Ki67 negative) was significantly increased compared to the control; something that I have also confirmed by FACS analysis (Figure 42A-C). Moreover, according to the previous result, the percentage of ciliated cells was also strongly increased (Figure 42B).



C

	FACS analysis		
	G0/G1	S	G2/M
CT	61,5	18,8	19,8
siGCP8 #1	81,8	5,6	12,6
siGCP8 #2	90,2	0,0	9,8
siKIF2A	79,6	13,6	6,8

Figure 42. KIF2A or GCP8 depletion stimulates cell cycle exit. A) RPE-1 cells were fixed 72h after control, GCP8 or KIF2A siRNAs transfection and stained for Ki67 and acetylated tubulin. DNA was labelled with DAPI. B) Quantification of percentage of ciliated cells. B) Quantification of the percentage of Ki67 positive or Ki67 negative cells and ciliated cells relative to the total number of cells counted per conditions (n=300 cells from three independent experiments, error bars s.e.m. C) Table with percentage of cells in each cell cycle phase from a FACS analysis.

10.1) GCP8 stimulates cell cycle exit in Cep83 KO cells

Occasionally, ciliated cells can be also seen in cycling cells and timely cilium disassembly has been linked to cell cycle progression (Kim S, 2011; Inoko A, 2012; Hidemasa Goto, 2013). To address whether ciliogenesis observed in GCP8 or KIF2A depleted cells was functionally related to cell cycle exit, I repeated the above experiment using an RPE-1 Cep83 knockout cell line (gift from Bryan Tsou laboratory). CEP83 is a distal appendage protein required for centriole membrane docking and ciliogenesis (Barbara E. Tanos, 2013). Therefore, in absence of GCP83, RPE-1 cells are not able to make cilia. As expected CEP83 knockout cells depleted of GCP8 did not assemble cilia. However, the number of cells negative for Ki67 staining was still significantly increased, suggesting that GCP8 depletion

promotes cell cycle exit independent of the ability of cells to assemble a primary cilium (Figure 43).

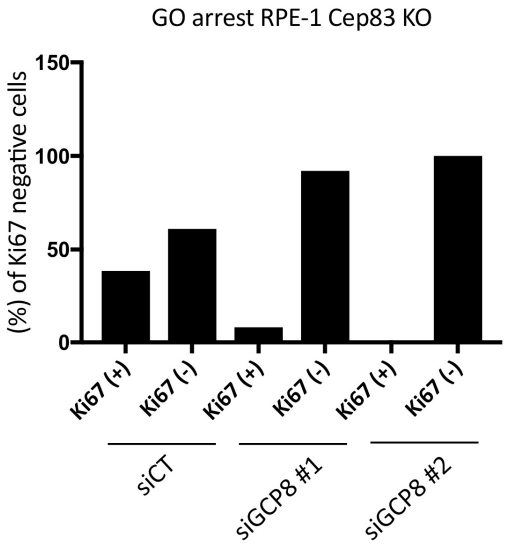


Figure 43. Cell proliferation effect on RPE-1 Cep83 knockout cells. RPE-1 Cep83 knockout cells were treated with control and GCP8 siRNA during 72h. The percentage of cycling cells was quantified using Ki67 antibody (n=100 cells from one experiment).

10.2) GCP8 or KIF2A depletion stimulate cell cycle exit in a p53 independent manner

The mechanism that triggers cell cycle exit after GCP8 or KIF2A depletion is still not fully understood. In HeLa cells disruption of the γTuRC typically impairs MT nucleation leading to mitotic arrested cells (Cota RR, 2016).By contrast, in absence of GCP8 cancer cells can undergo mitosis without any obvious defect (Neus Teixidó-Travesa, 2010). Moreover, a slight increase in the duration of mitotic progression has been shown to activate p53-dependent G1 arrest in RPE-1 cells (Uetake Y & Sluder G., 2010). Therefore, in order to investigate whether GCP8 depletion promotes cell cycle arrest by mildly delaying mitosis, I decided to analyse the effects on cell cycle progression in RPE-1 p53 knockout cells (gift from Andrew Holland laboratory). Interestingly, in absence of p53, GCP8 or KIF2A depletion also stimulate G0 arrest (Figure 44), suggesting that the effect on cell cycle progression does not come from mitotic problems.

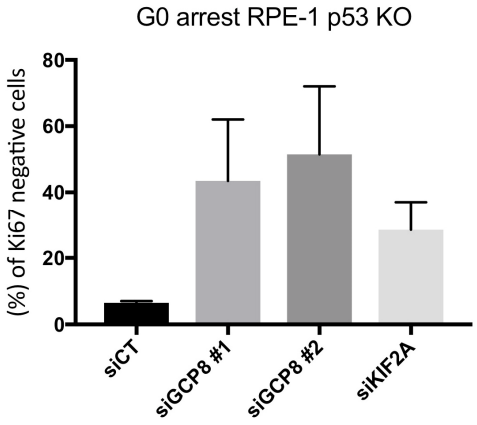


Figure 44. Cell proliferation effect on RPE-1 p53 knockout cells. RPE-1 p53 knockout cells were treated with control, GCP8 and KIF2A siRNA during 72h. The percentage of cycling cells was quantified using Ki67 antibody (n= 100 cells from 2 independent experiments, error bars s.e.m)

10.3) The effect on ciliogenesis is specific from GCP8

Due to the difficulties to rescue GCP8 depletion phenotypes, I decided to alternatively analyse the effect on RPE-1 cells after knocking down the levels of GCP8 with 3 additional siRNAs (Figure 45A). According to our hypothesis, ciliogenesis is the final consequence of disrupting GCP8 in RPE-1 cells. Therefore, and considering the simplicity of the assay, I decided to quantify the percentage of ciliated cells with the other siRNAs. Consistently, I observed an increase in the number of ciliated cells in all cases, confirming the specificity of the described phenotypes (Figure 45B).

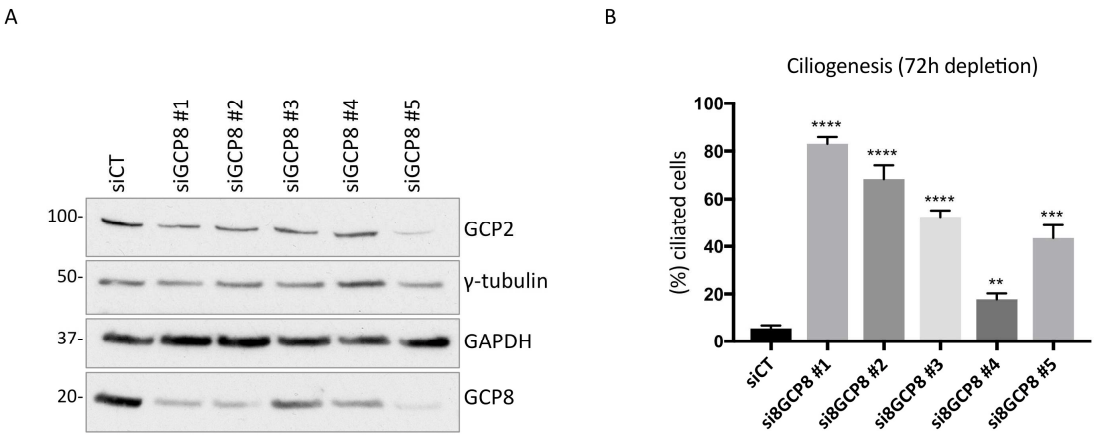


Figure 45. GCP8 depletion stimulates ciliogenesis with multiple siRNAs. A) Immunoblot against GCP2, γ-tubulin, GAPDH and GCP8 from RPE-1 cells depleted with 5 different GCP8 siRNAs. B) Quantification of percentage of ciliated cells (n=200 cells from at least two independent, error bars s.e.

11) Depletion of GCP8 or KIF2A reduces mTOR activity

Finally, we knew that GCP8 depletion stopped cell proliferation by arresting the cells in G0, although the mechanism that triggers cell cycle exit was still missing.

The mammalian target of rapamycin (mTOR) has been shown to regulate cell cycle progression by promoting p27 degradation (Fingar DC, 2004*,* Hong F, 2008, Poüs C & Codogno P., 2011). Moreover, it has been recently proposed that mTOR localizes at the Golgi apparatus, at least transiently, to be activated by Rheb, whose Golgi localization has been clearly shown (Gosavi P, 2018*;* Hao F, 2018). Hence, it may be possible that GCP8 and KIF2A affect mTOR signalling through their role in organizing the Golgi apparatus. To support this hypothesis, I performed a final experiment analysed by WB the levels of phospho-S6 ribosomal protein (pS6RP), which is a known mTOR downstream effector widely used to measure mTOR activity, and of p27 after depleting GCP8 or KIF2A. Indeed, I found that GCP8 or KIF2A depletion reduced mTOR activity and caused upregulation of p27 (Figure 46).

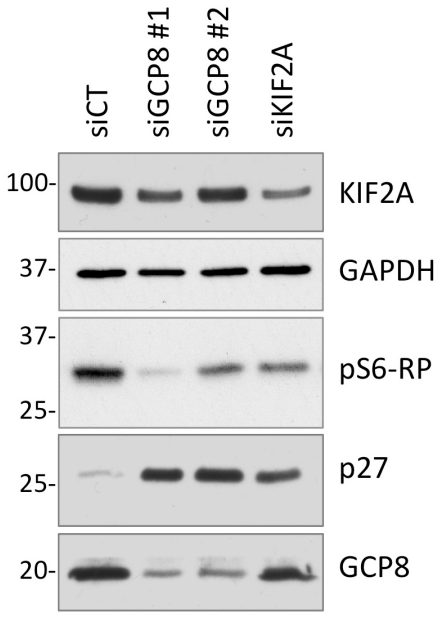


Figure 46. GCP8 or KIF2A depletion reduces mTOR activity. Immunoblot for KIF2A, GAPDH, phosho-ribosomal protein 6 (pS6-RP), p27 and GCP8 from in RPE-1 cell extracts after control, GCP8 or KIF2A siRNA transfection

DISCUSSION

In a previous study we described the cellular function of GCP8 in cancer cell lines such as HeLa or U2OS cells (Neus Teixidó-Travesa, 2010). These cells are useful for studying the role of MTs during mitosis, but they are not ideal for studying MT organization including by non-centrosomal mechanisms in interphase and the resulting cellular responses. By contrast, non-transformed, telomerase-immortalized RPE-1 cells are widely used for this purpose. Moreover, these cells display intact cell cycle arrest or exit responses to certain conditions and are able to form cilia, providing also the opportunity to analyse potential effects on cell cycle progression and ciliogenesis.

1) GCP8 regulates MT nucleation in interphase

We previously proposed that GCP8 regulates MT nucleation by promoting the recruitment of PCM proteins including γ-tubulin to the centrosome during interphase but not during mitosis (Neus Teixidó-Travesa, 2010). Moreover, contrary to the depletion of other γTuRC subunits, GCP8 depletion does not cause any significant mitotic arrest or delay, which is in agreement with a specific role of GCP8 during interphase. However, it should be noted that, to confirm this conclusion, live cell imaging of GCP8 depleted samples would be necessary to discard any mild effect on mitotic progression.

Surprisingly I found that in RPE-1 cells MT nucleation at the centrosome is increased in the absence of GCP8 under conditions where the centrosomal levels of γ-tubulin are not affected. This effect was dependent on the RNAi target sequence. A reduction of nucleation, as we previously published for cancer cell lines, was only observed when centrosomal GCP8 was most efficiently depleted, leading to a reduction in the recruitment of γ-tubulin. How can this puzzling result be explained?

One possibility might be the presence of multiple GCP8 isoforms and that the isoform expression pattern differs among cell lines. In this regard, I have identified by WB two additional GCP8 isoforms, apart from the main one that runs at 20kDa, expressed at variable levels in RPE1, Hela and U2OS cells. The most complete depletion of all GCP8 isoforms at the centrosome might impair γTuRC centrosomal localization and thus nucleation. Incomplete centrosomal depletion of all isoforms, on the other hand, may allow

stabilization of centrosomal γTuRC but relieve the inhibitory activity provided by canonical GCP8.

2) Identification of new GCP8 binding proteins

To understand how GCP8 may negatively regulate nucleation I screened for novel interactors. Protein-interactions that occur transiently or only at specific cellular structures can be difficult to detect by conventional affinity purification methods. Complementary proximity labelling approaches, such as the BioID, are recently commonly used to overcome these limitations. However, due to the different nature of both techniques, the interpretation of combined results is usually not an easy task. The BioID provides information only about proteins that are in close proximity or that transiently interact with the protein of interest under physiological conditions. By contrast, the FLAG pull down allows to identify direct and indirect interactors including entire protein complexes.

In agreement with this observation, the percentage of proteins that were identified with both complementary methods was relatively low. In total, I obtained more than 500 protein candidates from both approaches. In order to evaluate only the top hits, I considered those with the highest SAINT value. Then, I decided to focus my search on proteins with a potential role in MT nucleation. Among the remaining candidates, the kinesin-13 protein KIF2A was significantly enriched in the FLAG pull down data. Thus, considering that it has been previously proposed that MCAK, another member of the kinesin-13 protein family, negatively regulates MT nucleation in vitro (Wieczorek M, 2015), I decided to analyse the role of KIF2A in this process in RPE-1 cells.

3) KIF2A negatively regulates MT nucleation

γTuRC alone is a poor MT nucleator in vitro and it has been proposed that in cells it requires specific activation to efficiently nucleate MTs. However, new evidences suggest that the formation of new MTs can also be actively suppressed by inhibiting γTuRC activity (Wieczorek M, 2015; Shen Y, 2017).

Interestingly, I have shown that this MT depolymerase, apart from its known role in the regulation of the MT plus end, inhibits MT nucleation in cells. Hence, one possibility is that

GCP8 depletion stimulates MT regrowth by impairing the recruitment of KIF2A to the γTuRC.

Furthermore, I mapped by co-immunoprecipitation the KIF2A binding region at the C-terminal half of GCP8. Thus, regarding the differences in the MT nucleation capacity between the two GCP8 siRNAs that I have commented on before, an alternative explanation may be that different isoforms bind KIF2A with distinct efficiencies. Hence, while the oligo 2 potentially depletes all GCP8 variants, oligo 1 may only compromise the interaction with KIF2A but not with γTuRC, for example by not targeting isoforms that lack or have alterations in their C-terminal part.

Moreover, I have shown that depletion of GCP8 reduces the levels of centrosomal KIF2A, as expected if GCP8 participates in its recruitment to the γTuRC. KIF2A has been reported to localize at the sub-distal appendages and the proximal end of the centrioles (Gregory Mazo, 2016*;* Tatsuo Miyamoto K. H., 2015), while GCP8 co-localizes with other γTuRC subunits at the PCM (Neus Teixidó-Travesa, 2010). However, according to my own results, GCP8 and KIF2A partially co-localize at the proximal end of the centriole, suggesting that there may be distinct pools of GCP8 and γTuRC at the centrosome that differ in their interaction with regulators.

4) GCP8 and KIF2A stimulate Golgi nucleation

Curiously, an interesting result from our mass spectrometry data was the identification of the Golgi protein GOLGA2/GM130. Although it was not one of the top candidates (based on its SAINT value), GM130 was one of the few proteins with both methods FLAG pull down and BioID. Thus, it would be interesting to explore whether GCP8 is associated with GM130 at the Golgi and whether GM130 may be involved in GCP8 recruitment.

Since RPE-1 cells have been widely used to study Golgi nucleation, I decided to evaluate the role of GCP8 and KIF2A in the formation of new MTs at this organelle. Interestingly, in this case I observed an increase in the number of MT growing from the Golgi in all the conditions including with both GCP8 siRNAs. Thus, contrary to the described effect on centrosome nucleation with the oligo2, Golgi nucleation is stimulated under the same

conditions. This observation suggests that the depletion of GCP8 with oligo 2, rather than affecting the integrity of γTuRC, may specifically impair its localization at the centrosome but not at the Golgi apparatus. This would be consistent with the slight reduction in the levels of γ-tubulin at the centrosome in cells treated with this oligo.

Moreover, I have shown that GCP8 partially co-localizes at the Golgi apparatus, similar to what has been previously described for γ-tubulin (Rosa M.Ríos, 2004). However, further knock-down experiments are required to confirm this localization.

5) GCP8 and KIF2A contribute to maintain Golgi integrity

Interestingly, not only the nucleation capacity but also the overall Golgi structure was impaired in GCP8 or KIF2A depleted cells, seemingly condensed and collapsed around the centrosome. This raises the question whether nucleation might contribute directly to maintenance of the Golgi ribbon.

The morphology of the Golgi is controlled by the MT cytoskeleton. Curiously, smaller and collapsed Golgi has been only reported under conditions where Golgi nucleation is inhibited but not stimulated as in my study (Yang C, 2017*;* Hurtado L, 2011*;* Shen Y, 2017). The change in the Golgi morphology is probably mediated by dynein, which moves Golgi compartments towards the centrosome. If both centrosomal and Golgi nucleation activities are stimulated, or if Golgi nucleation is selectively inhibited without affecting centrosomal nucleation, this may result in an increased transport of Golgi compartment to the centrosome.

Additionally, a possibility that remained unexplored is that GCP8 may regulate the integrity of the Golgi in a γTuRC-independent manner that may not depend on MT nucleation. Since I mapped the binding region of GCP8 to the first 24 amino acids of GCP2, I tried to reproduce the GCP8 depletion phenotypes by simply overexpressing the GCP2Δ24 construct. However, since even with the wild type GCP2 I observed a slight reduction in centrosomal nucleation, I concluded that these constructs may not be functional.

Moreover, considering the stoichiometry of the complex, the overexpression of the GCP2 mutant construct may not be sufficient to compete with the endogenous protein, which is present in multiple copies in the γTuRC. To address this rescue experiments would be required. In fact, for another study I used a similar approach to impair the binding of MZT1 to the γTuRC by expressing an RNAi-resistant MZT1-binding mutant of GCP3 after knocking down the endogenous GCP3. Therefore, I am currently working on rescue experiments regarding GCP8 and GCP2, to obtain more mechanistic insight into the role of GCP8 in the regulation of MT nucleation and Golgi integrity.

Besides that, although my results are still preliminary, I have shown that the co-depletion of GCP8 and GCP2 restores both centrosome nucleation and Golgi morphology, suggesting that, at least these phenotypes depend on the γTuRC. Moreover, I have seen that both GCP2 depletion and overexpression strongly affect GCP8 expression levels, suggesting that GCP8 is stabilized through its binding to GCP2 and thus this interaction is likely relevant for GCP8's cellular function.

6) GCP8 and KIF2A regulate cell cycle progression

Even though no effect of GCP8 depletion has been described during mitosis (Neus Teixidó-Travesa, 2010) one interesting observation was that cells lacking GCP8 were smaller, rounder and seemed to stop proliferating. Therefore, I analysed the consequences of depleting GCP8 or KIF2A on cell cycle proliferation. Strikingly, I observed an increase in the percentage of cells that exit the cell cycle and form a cilium.

The defects in the Golgi structure may explain the effects of GCP8 or KIF2A depletion in cell cycle exit and ciliogenesis. Since many Golgi-associated proteins are involved in ciliary processes (David Asante, 2013; John A. Follit, 2006), an abnormal nucleation at the Golgi apparatus may result in problems in trafficking of ciliary proteins. Moreover, it was not clear whether the observed increase in ciliogenesis was the cause or the consequence of the G0 arrest. In this regard, I concluded that the effect on ciliogenesis was triggered by

cell cycle exit since I also observed a similar effect on cell proliferation in RPE-1 cells that were not able to form a cilium by knockout of the centriole distal appendage protein Cep83.

Besides this, despite primary cilia being assembled, it would be worth investigating whether ciliary signalling is functional after GCP8 or KIF2A depletion, an issue that has not been explored during my thesis.

7) GCP8 and KIF2A control cell proliferation through mTOR

What triggers cell cycle exit in cells lacking GCP8 or KIF2A? Key regulators of cell cycle progression are the cyclin-CDK protein complexes (Paul Nurse & Pierre Thuriaux, 1980*;* Evans T, 1983*;* Weinert TA & Hartwell LH., 1988). These complexes can be regulated at different levels including activation or inactivation by phosphorylation/dephosphorylation and association to cyclin-dependent kinases inhibitor (Sherr CJ & Roberts JM., 1999).

The activation of the p53-p21 pathway is one of the best-characterized responses to several cellular problems, including DNA damage, cytokinesis failure or mitotic delay (Hayashi MT & Karlseder J, , 2013). However, none of the mentioned conditions have been reported for GCP8 depleted cells. While cells cannot undergo mitosis in absence of the γTuRC, the depletion of GCP8 does not cause mitotic arrest, although one cannot rule out very mild defects that may not cause arrest but only slightly slowed down mitotic progression. Such as small effect may be difficult to detect by quantifying the percentage of mitotic cells but might be sufficient to activate the p53-p21 pathway. In fact, it was recently shown that a mitotic delay of 30 min (2h vs 90 min in controls) is sufficient to trigger subsequent p53-dependent G1 arrest in RPE-1 cells (Uetake Y & Sluder G., 2010). Therefore, I decided to evaluate this issue after GCP8 or KIF2A depletion in p53 knockout RPE-1 cells. Strikingly, GCP8 or KIF2A depletion also stimulates G0 arrest in these cells, suggesting that this response does not depend on p53.

Apart from the p53 pathway, another cellular mechanism that acts on cell cycle progression is controlled by mTOR, which senses the nutrient stage of the cell and regulates p27 function (Toker, 2008*;* Hong F, 2008). Previous work showed that KIF2A stimulates mTOR activity by promoting lysosome positioning in the cell periphery *(*Korolchuk VI, 2011*;* Elma

Zaganjor, 2014), but effects on cell cycle progression were not investigated in these studies. Moreover, it was proposed that mTOR localizes at the Golgi apparatus or at least must be in close proximity to this organelle for its activation by Rheb (Charles Betz & Michael N. Hall, 2013*;* Gosavi P, 2018). Hence, I decided to evaluate whether cell cycle exit after GCP8 depletion may involve regulation of mTOR activity. Indeed, I have shown by WB that the activity of mTOR is significantly reduced after GCP8 RNAi. Curiously, oligo 1 most strongly reduced mTOR activity, whereas oligo 2 and KIF2A siRNA produced milder effects. By contrast, p27 was similarly increased in all conditions. A possible explanation may be that, although p53 is not essential to stimulate cell cycle exit after GCP8 or KIF2A depletion, it may partially contribute and promote G1 arrest in wild type cells, due to a potential mild mitotic delay after treatment with oligo 2 or KIF2A siRNA. To confirm this hypothesis, it would be essential to analyse the effect on mTOR activity in a p53 knockout context.

Altogether, my data suggest that GCP8 negatively regulates MT nucleation by impairing the binding of KIF2A to the γTuRC. The increase in the centrosomal and Golgi nucleation alters the normal MT organization throughout the cell. This leads to problems not only in the morphology but also in the integrity of the Golgi apparatus. A compromised Golgi is defective for activating mTOR signalling, either directly or indirectly through lysosome positioning, which stops cell proliferation and promotes G0 arrest (Figure 47).

Hence, I described an unexpected model by which MT nucleation can regulate interphase cell cycle progression through mTOR.

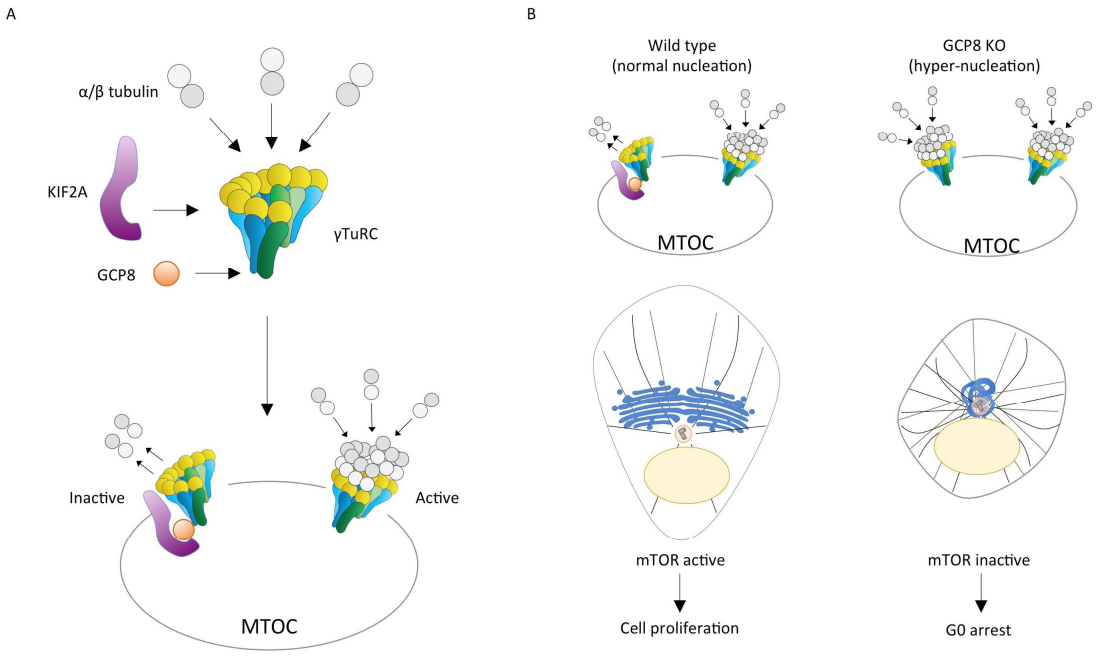


Figure 47. Model for the role of GCP8 in the regulation of MT nucleation and cell cycle progression. A) In normal conditions, some γTuRC complexes at the MTOCs remain inactive by binding GCP8 and KIF2A, while other complexes do not. B) In the presence of GCP8, KIF2A binds to the γTuRC and limits MT nucleation events in the cell. On the other hand, γTuRC without GCP8 can normally nucleate MTs. When MT nucleation is well controlled, the Golgi ribbon acquires its prototypical conformation allowing the activation of mTOR. By contrast, unconstrained MT nucleation gives rise to an abnormal formation of new MTs, which alters the equilibrium of MT organization. The excess of MTs compromises the integrity of the Golgi ribbon, preventing the activation of mTOR, and resulting in cell cycle exit.

CONCLUSIONS

- GCP8 binds to the γTuRC through the first 24 amino acids of GCP2
- The overexpression of GCP2Δ24 reduces GCP8 localization at the centrosome
- The depletion of GCP8 compromises cell morphology and affects MT organization
- GCP8 depletion increases centrosome regrowth in RPE-1 cells under conditions where other γTuRC components are not affected
- Identified KIF2A as a new GCP8 interacting protein
- The C-terminal region of GCP8 is required for binding KIF2A
- GCP8 depletion reduces the levels of KIF2A at the centrosome
- KIF2A depletion stimulates MT regrowth at the centrosome in RPE-1 cells
- Expression of GCP8 does not affect MT regrowth at the centrosome in RPE-1 cell
- The disruption of MZT1-γTuRC binding impairs the binding of the activator motif CM1 and the localization of the complex at the centrosome
- GCP8 or KIF2A depletion stimulates Golgi MT regrowth
- GCP8 or KIF2A depletion compromises Golgi integrity
- GCP8 or KIF2A depletion stimulates cell cycle exit in a p53 independent manner
- GCP8 or KIF2A depletion reduces mTOR activity

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