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Lindström, Lisa

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LISA LINDSTRÖM
FACULTY OF MEDICINE | LUND UNIVERSITY

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Lisa Lindström

LUND
UNIVERSITY

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Abstract

Cancer is a heterogeneous disease and treatment regime is dependent on type and location of the tumor. Several of the commonly used chemotherapeutics target the functions of the microtubules. One of the major problems with these drugs is the adverse effects associated with their use. Another problem many of the chemotherapeutics today face is resistance. Therefore there is a constant need for development of new drugs in the fight against cancer. Many tumors achieve independent growth by carrying mutations in the retinoblastoma (pRB) signaling pathway. It has been shown that reduced protein levels of nuclear gamma-tubulin in tumor cells lacking pRB expression induce cell death. Consequently inhibiton of gamma-tubulin might be used as a new strategy for therapy of pRB-negative tumors with fewer side-effects.

The aim of this thesis was to evaluate gamma-tubulins' potential as a novel therapeutic target in the treatment of tumors with a deregulated pRB and to characterize the gamma-tubulin meshwork. We searched for compounds that specifically interfere with the nuclear activity of gamma-tubulin and the effect of the substances were studied in silico, in vitro and in vivo. Among the tested substances we found one, citral dimethyl acetal (CDA), that was specific for gamma-tubulin. CDA caused cell death in cell lines with non-functional pRB and reduced tumor growth in a mice xenograft model. We also showed that CDA interact with gamma-tubulin at the amino acid cysteine 13 at the GTPase domain of gamma-tubulin. Our results demonstrate that it is possible to develop new chemotherapeutics that target the nuclear activity of gamma-tubulin and give rise to fewer side-effects.

We also observed that gamma-tubulin form a meshwork in cells that connects the nuclear and cytoplasmic compartments. The network is important in mitosis during formation of the nuclear envelope (NE) where it is responsible for recruitment of the lamina to the chromatin. We also observed that gamma-tubulin form cytosolic tubules together with several other proteins, including GCP2, GCP3, GCP6 and pericentrin. The gamma-tubulin meshwork also interacts with the mitochondria. In addition, we provide evidence for that the gamma-tubulin GTPase domain is involved in regulation of mitochondria organization and homeostasis.

Key words Gamma-tubulin, retinoblastoma, nuclear formation, meshwork, mitochondria, homeostasis

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The gamma-tubulin meshwork as a therapeutic target

Lisa Lindström

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Till Jesper och min familj

Content

List of papers

The thesis is based on the following papers, referred to in the text by their Roman numerals.

Paper I

Lindstrom L, Villoutreix BO, Lehn S, Hellsten R, Nilsson E, Crneta E, Olsson R, Alvarado-Kristensson M.

Therapeutic targeting of nuclear gamma-tubulin in RB1 negative tumors.

Mol Cancer Res 2015 **13**(7): 1073-1082

Paper II

Rosselló CA, **Lindstrom L**, Glindre J, Eklund G, Alvrado-Kristensson M.

Gamma-tubulin coordinates nuclear envelope assembly around chromatin.

Heliyon 2016 **2**(9): e0016

Paper III

Lindstrom L, Eklund G, Alvarado-Kristensson M.

Characterization of gamma-tubulin filaments in mammalian cells.

Submitted

Paper IV

Lindstrom L, Nilsson H, Alvarado-Kristensson M.

The GTPase domain of gamma-tubulin controls mitochondria homeostasis.

Submitted

No reprint permissions was required for paper I and II.

Abbreviations

ATP	Adenosine triphosphate		INK4	Inhibitor of cyclin-dependent kinase 4
BRCA1	Breast and ovarian susceptibility protein		Kip	Kinase-inhibitory protein
CDA	Citral dimethyl acetal		M	Mitosis
CDKs	Cyclin dependent kinases		MDM2	mouse double minute 2 homolog
CKIs	CDK inhibitors		MOZART	Mitotic spindle organizing proteins associated with a ring of gamma-tubulin
Cip	CDK-inhibitory protein			
CycD	Cyclin D			
CycE	Cyclin E		mtDNA	Mitochondria DNA
CycA	Cyclin A		MTCO2	Cytochrome c oxidase subunit II
CycB	Cyclin B			
Cys13	Cysteine 13		MTOC	Microtubule organizing center
C53	cdk5 activator-binding protein		NE	Nuclear envelope
DMF	Dimethyl fumarate		NPC	Nuclear pore complex
DNA	Deoxyribonucleic acid		OCR	Oxygen consumption rate
E2Fs	E2F transcription factors		p53	cellular tumor antigen p53
G_1	Gap phase 1		pRB	Retinoblastoma protein
G_2	Gap phase 2		RAD51	DNA repair protein 51
GCP	Gamma-tubulin complex protein		*RB1*	Retinoblastoma gene
			R-point	Restriction point
GDP	Guanosine diphosphate		S	Synthesis phase
GTP	Guanosine triphosphate		*TUBG1*	Tubulin gamma 1 gene
γTuSC	Small gamma-tubulin complex		*TUBG2*	Tubulin gamma 2 gene
γTuRC	Gamma-tubulin ring complex			
HDAC	Histone deacetylase			

Introduction

According to WHO, in 2015 1 out of 6 deaths worldwide were caused by cancer and in Sweden the second leading cause of death among both males and females was a result of tumor disease (1, 2). Tumor disease is an extremely heterogeneous disease composed of a number of different malignancies. Therefore correct and early diagnosis is important, as every type of cancer requires a specific treatment routine such as surgery, radiotherapy or chemotherapy. There is a need for new and better-tolerated chemotherapeutics as many chemotherapeutics today are facing an increasing problem with side effects and resistance that impede their use.

In this thesis, the overall aim has been to evaluate the protein gamma-tubulin as a potential drug target in cancer and to find novel substances that target the gamma-tubulin pathway in tumors with a non-functional retinoblastoma pathway. During the course of the project we have also found it necessary to further characterize and evaluate novel cellular functions of gamma-tubulin. In the studies this thesis is based on, we have mostly used *in vitro* methods but we have also studied drug efficacy *in vivo* in a mice xenograft model. Previous work in our group have shown that there is an inverse correlation between the expression levels of retinoblastoma protein (pRB) and gamma-tubulin in several tumors and that reduced levels of gamma-tubulin in cells with impaired pRB function cause cell death while leaving the normal cells of the body unharmed (3).

Taken as a whole, my thesis aims to better understand different cellular functions of gamma-tubulin and how gamma-tubulin can be targeted in different ways to develop new drugs against cancer. Hopefully, our findings can contribute to the development of better-tolerated and safer drugs in the future.

Brief Introduction to Tumor Biology

Cancer is a general term used to describe more than 100 different diseases that all have in common a defect in their normal cell proliferation machinery. Tumorigenesis is a multistep process that is characterized by genomic instability, deregulated proliferation and an inflammatory state (4, 5). Genomic alterations can arise from mutations or germ line alterations, which bestow a growth advantage on the cell enabling them to eventually take over the local tissue. However, spontaneous mutations are relatively rare due to the ability of the genome maintenance system to detect and correct defects in the DNA. Other mechanisms to achieve genomic alterations are through for example, epigenetic changes through histone modifications and DNA methylation (6).

The genes affected by these mutations can be divided into two classes, proto-oncogenes and tumor suppressor genes (7–9). Once the proto-oncogene is mutated it becomes an oncogene and cause a normal cell to become cancerous. Common to most oncogenes is the increase in production of proteins involved in cell proliferation, cell differentiation and cell death (7). Mutations to proto-oncogenes are often called gain of function mutations and cause increased expression levels or activity of the proto-oncogene.

On the other hand, tumor suppressors act to control inappropriate cell growth and division to stop cells before they become cancerous. Tumor suppressor genes are also involved in DNA repair mechanisms and cell death. Loss of tumor suppressor gene function is therefore often critical and speed up the transformation of a normal cell to a cancer cell (9). Mutations in tumor suppressor genes are recessive which means that both gene copies in a cell must be mutated for the cell to become cancerous. This phenomenon is known as the "two-hit" hypothesis and was first proposed in the 1970s by Alfred Knudson after his studies of the childhood cancer retinoblastoma (10). However, the actual gene responsible for causing retinoblastoma, *RB1* gene, was not discovered until 1986 by Friend and colleagues (11). The *RB1* gene product, pRB, was later found to be involved in regulation of cell cycle, differentiation and apoptosis (12, 13) and these functions will be described in more detail later in thesis.

Ultimately the combined effect of these processes leads to the transformation of a normal cell into a malignant cell. It was suggested by Hanahan and Weinberg that the majority of genomic alterations seen in cancer is a manifestation of several

changes in cell physiology, known as the hallmarks of cancer and they are defined as follows (4, 5):

- **Sustained growth signals.** There are several ways in which cancer cells can achieve a sustained growth signaling. They can for example produce their own growth signals, mitogens, or up-regulate the receptors that mitogens activate.

- **Decreased sensitivity to anti-growth signals.** Normally there are programs within a cell that suppress proliferation. In cancer cells these programs are often deregulated.

- **Evasion of apoptosis.** The mechanisms by which cancer cells can evade apoptosis are many, probably as a result of the assortment of apoptosis inducing signals. The most common strategy is deregulation of pro- and anti-apoptotic signals.

- **Unlimited proliferation**. A normal cell can only undergo a predefined number of divisions. Therefore, cancer cells acquire traits that allow them to multiply unlimited.

- **Up-regulation of angiogenesis.** Cancer cells need a constant supply of nutrients and oxygen and removal of waste products, which they obtain by a constant activation of angiogenesis to form new blood vessels.

- **Invasion of tissue and metastasis.** Is important for the malignant cell to allow cells from the primary tumor to escape and colonize adjacent and distant locations within the body and form metastases.

- **Reprograming of cellular energetics.** Many cancer cells reprogram their metabolism so they rely on glycolysis even in the presence of high oxygen levels, a process known as aerobic glycolysis.

- **Evading the immune response.** It was originally thought that cancer cells need to avoid detection by the immune response to form and that cancer cells deactivate the immune system. However, in recent years the picture has become more complicated as we now know that tumor cells also activate certain immune cells.

As highlighted in the previous section it is not a single adverse event that leads to the formation of a malignant cell. On the contrary, for a cell to become malignant it needs to acquire the traits mentioned above. The focus of this thesis will be on cell cycle and metabolic changes cells acquire during malignant transformation and how they are targeted during chemotherapeutic treatment.

The Cell Division

There are two different types of cell division; asymmetric and symmetric cell division. In asymmetric cell division a cell is divided into two cells with different cellular content (14, 15). This process is important during embryonic development as well as during differentiation in adult tissue. During symmetric cell division the components of the mother cell are duplicated and divided into two identical daughter cells (16). This process is tightly controlled and characterized by oscillating levels of regulatory proteins that integrate external and internal signals to regulate cell division.

The process of cell division is typically divided into four main phases two gap phases, the synthesis phase and mitosis and the completion of all four phases is called the cell cycle, which is shown in figure 1. In the first gap phase, G1, the cell grows in size and prepares for the next phase, the synthesis phase, S. During S phase the genetic material is replicated simultaneously with the centrosomes, the organelle responsible for parting the replicated chromosomes (17, 18). Once the genetic material is duplicated the cell enters a second gap phase, G2. During G2 the cell assures that there is a duplicate of each cellular component. Finally, during mitosis or M phase the cellular contents are divided into two daughter cells. There is a fifth reversible non-proliferating state in the cell cycle, G0, which cells in G1 can enter. During the cell cycle the cells monitor the completion of each phase and make sure it is prepared for the upcoming events, this type of control is called a checkpoint (19, 20). In case of errors, a checkpoint can delay cell division, giving the cell a chance to correct possible flaws (20). As cell cycle deregulation is a common trait in cancer, studying the normal process of cell division is important to understand the mechanisms behind cancer formation and progression.

Cell cycle associated proteins

Normally, cell division is a tightly controlled process that is necessary for maintaining normal tissue homeostasis and involves several proteins. In this section the central proteins involved in cell cycle progression and regulation will be discussed as well as some key concepts during cell division.
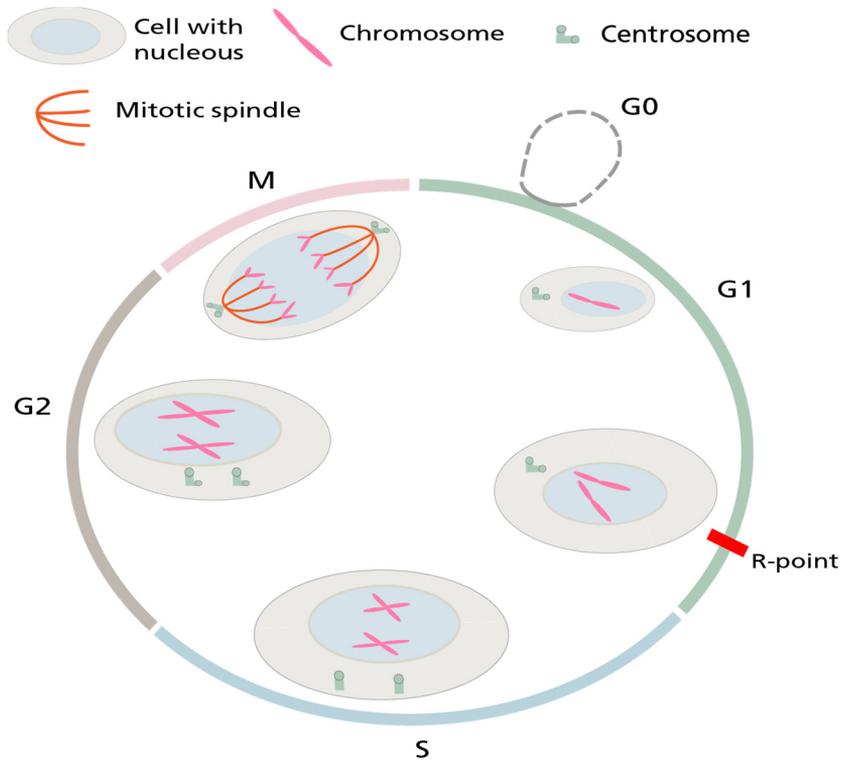
Figure 1. The cell cycle phases.
The process of cell division is divided into four phases. During the first phase, G1, the cell grows in size and prepares for the upcoming S phase. In S phase the DNA (pink) and the centrosome (green) are duplicated. During G2 the cell ensures that the previous phases are completed and prepares for mitosis, M. In the last phase of the cell cycle, M, the chromosomes are segregated by the mitotic spindle (orange) and two daugther cells are formed. (Modified from Abubakar et al 2008 and Pike and Fisk 2011 (21, 22)).

Cyclins and cyclin-dependent kinases

The progression through the various phases in the cell cycle is controlled by proteins known as cyclin-dependent kinases (CDKs) (23, 24). CDKs belong to a family of serine/threonine protein kinases that activate downstream targets through phosphorylation (a posttranslational modification process that adds phosphorus groups to a protein). To become active CDKs need to bind to regulatory proteins known as cyclins. Different CDK-cyclin complexes are present at different phases of the cell cycle. In contrast to CDK protein levels, which are constitutively expressed throughout the cell cycle, the levels of cyclins fluctuate. In this way the cell ensures that the appropriate CDKs are activated in the proper cell cycle phase.

16

As a response to mitogenic signals during G1 the D-type cyclins (CycD) are expressed and bind to and activate CDK4 and CDK6. Activation of CycD-CDK4/6 complex causes a partial phosphorylation and inactivation of the downstream target, the pRB (24, 25). pRB is further phosphorylated and completely inactivated by the activity of cyclin E-CDK2 complex. Inactivation of pRB causes the release of the E2F transcription factors (E2Fs), which are essential for the transition from G1 to S phase (26, 27). The inactivation of pRB and subsequent release of E2Fs mark the point of no return in G1 known as the restriction point, the R-point. After this stage the cell is committed to division and no longer respond to external stimuli. For the cell to progress through S phase cyclin A (CycA) needs to activate CDK2 and in late G2 cyclin A activates CDK1 for the transition to mitosis (23). Progression through mitosis is regulated by CDK1 in complex with cyclin B (CycB) (23). In figure 2, a schematic illustration of the proteins involved in progression through the cell cycle phases are shown.

Due to the proliferation-promoting properties of cyclins, they have been described as proto-oncogenes and overexpression or deregulation of both cyclin D1 and cyclin E is common in cancers (26). pRB on the other hand is considered a tumor suppressor and is often inactivated in cancer (12, 28).

Inhibitors of the cell cycle

Not only fluctuating levels of cyclins control the activity of the CDKs. In addition, the activity of CDKs is under the control of inhibitory proteins known as CDK inhibitors (CKIs) and subcellular localization of the proteins (29). The CKIs can be divided into two distinct families, the INK4 family and the Cip/Kip family, based on mechanism of action and to which CDKs they bind (29).

The INK4 family is composed of four different proteins $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, $p19^{INK4d}$ that are highly conserved among different species (30). The INK4 family inhibitors exert their control on cell cycle by hindering the formation of active cyclin-CDK complexes (30, 31). They specifically inhibit CDK4 and CDK6 by binding to their catalytic subunits, arresting the cell in G_1 (32)(33).

The three members of the Cip/Kip family of CKIs are $p21^{CIP1/WAF1}$, $p27^{KIP1}$, $p57^{KIP2}$. The Cip/Kip type CKIs also bind to cyclin-CDK complexes and can interact with all cyclin-CDK complexes (29, 34, 35). The Cip/Kip proteins act as negative regulators on cyclin E and cyclin A dependent kinases, but positively regulate the cyclin D dependent kinases (29). Due to their ability to hinder cell cycle progression, CKIs are considered to be tumor suppressors and deregulation is not uncommon in cancer (29, 36).
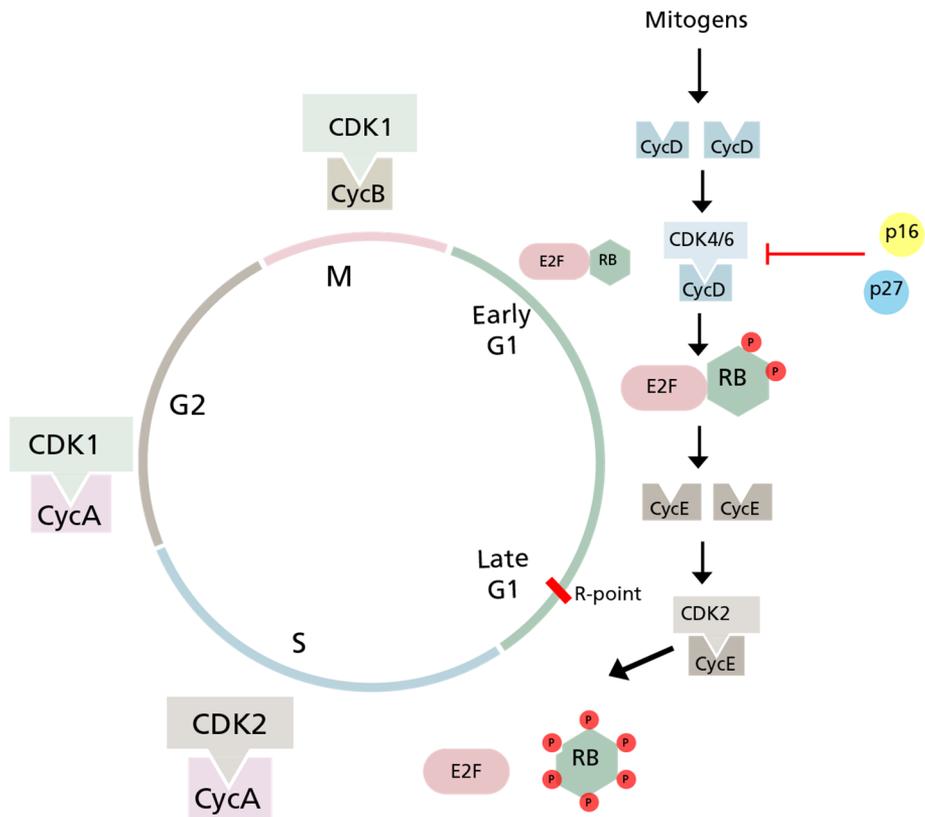
Figure 2. Fluctuations of cyclin dependent kinases and cyclins during cell cycle.
During early G1, Cyclin D activates CDK4 and CDK6 in response to mitogens. Activation of CDK4/6 leads to a partial phosphorylation of retinoblastoma protein (pRB) and to the subseqent transcription of cyclin E (CycE). Then CycE forms a complex with CDK2, which further phosporylates pRB. The inactivation and subsequent release of E2Fs is referred as the restriction point (R-point), and causes S phase entry. S phase progression is driven by cyclin A (CycA)- CDK2 complex. In G2 CycA associate with CDK1, but in M phase CDK1 is activated by Cyclin B. (Modified from Giacnti and Giordano 2006 (28)).

Retinoblastoma

As mentioned earlier RB gene was the first tumor suppressor described and its gene product, pRB, is involved in many processes ranging from cell cycle and apoptosis to cell differentiation (12, 13). The RB gene is located on chromosome 13q14 and belongs to a family of proteins known as the "pocket proteins" (37). The protein family also includes p107 and p130 (28, 37, 38) and these proteins are characterized by a conserved domain, the pocket, which contains two distinct binding sites. The first binding site interacts with proteins containing a LXCXE-

motif, such as histone deacetylases (HDAC), to repress transcription (12, 39, 40). The second binding site is necessary for binding to the activators of transcription E2F1, E2F2 and E2F3 (40, 41). The resulting RB-HDAC-E2F complex can repress transcription by binding to DNA and inactivate the expression of E2F controlled genes.

RB and cell cycle progression

During cell division, phosphorylation of pRB is necessary for progression through the cell cycle. During G1, pRB is initially present in an unphosphorylated active state. In this state RB forms a transcriptional repressive complex together with HDAC and E2F (39). As the cell receives mitogenic stimuli and progress through G1, pRB will be hypophosphorylated by cyclin D-CDK4/6 complex and partially inactivated (24, 28). According to a model proposed by Harbour et al. the partial phosphorylation by cyclin D-CDK4/6 causes HDAC to dissociate from pRB. The displacement of HDAC is enough to relieve the transcriptional repression of the cyclin E gene that is required for further cell cycle progression (24). Increased protein levels of cyclin E lead to the formation of cyclin E-CDK2 complex, which inactivates pRB by hyperphosphorylation. Hyperphosphorylation of pRB results in a conformational change of the pocket region, resulting in the dissociation of the pRB E2F complex and subsequent activation of genes involved in S-phase progression (24).

RB and apoptosis

It has been shown that loss of pRB function can trigger p53 dependent apoptosis possibly as an intrinsic mechanism to eliminate cells with a dysfunctional pRB (42, 43). In a normal cell, p53 is present at low levels due to formation of an autoregulatory loop with the protein mouse double minute 2 homolog, MDM2 (a negative regulator of p53). p53 activates the expression of MDM2 that binds to p53 and inhibits p53 activity in several ways (44, 45). In cells with dysfunctional pRB the levels of free E2F1 increases, which is known to directly activate expression of p14[ARF]. In 1998 Bates et al showed that p14[ARF] binds to the MDM2-p53 complex and inactivates MDM2 thus preventing p53 degradation (46). The resulting accumulation of p53 causes cell cycle arrest or apoptosis, protecting the cell from improper cell division.

Increased protein levels of free E2F, due to for example loss of pRB, can also cause apoptosis by a p53 independent pathway. The mechanisms in this pathway are less well studied but are thought to involve down-regulation of anti-apoptotic genes (13). It has also been shown that high levels of free E2Fs can increase caspase expression, which increase the likelihood that a cell will enter apoptosis (47).

RB and cancer

Due to its importance in regulation of cell cycle progression, the pRB pathway is often disrupted in various malignancies including osteosarcoma, the childhood cancer retinoblastoma, melanoma, breast cancer, bladder cancer, colon cancer, neuroblastoma, prostate cancer and small cell lung cancer (12, 48, 49). Disruptions in the pRB pathway include among others deletion of the RB gene, mutational inactivation, hyperphosphorylation, loss of function due to inhibition by viral oncoproteins and by deregulation of components upstream of pRB in the pathway (50, 51). The direct consequence of all of these routes is an increase of free E2Fs and unrestrained progression through the cell cycle, which result in formation of a malignant cell.

Mitosis, nuclear envelope and DNA segregation

During mitosis the goal is to accurately separate the duplicated DNA into two daughter cells, while maintaining genomic integrity to avoid possible formation of malignant cells. Mitosis can be further divided into four distinct phases basesed on the state of DNA separation, as shown in figure 3.

- **Prophase.** The chromatin is condensed and forms into chromosomes and the nuclear membrane starts to dissolve. Also, the two centrosomes are separated to opposite poles of the cell and form the mitotic spindle that start to interact with the chromosomes.

- **Metaphase.** The chromosomes are attached to the mitotic spindle and align in the metastatic plan, also called the equator, in between the two centrosomes.

- **Anaphase.** The centromere, the structure that links the sister chromatids with each other in the chromosomes, are divided and the sister chromatids are moved to opposite poles of the cell by the shortening of the microtubules. In late anaphase the poles separate from each other.

- **Telophase.** A nuclear membrane forms around each set of chromatids and the cell divides into two daughter cells. Also the spindle microtubules depolymerize and the chromatin decondenses.

Nuclear envelope and nuclear lamina

To facilitate the separation of the sister chromatids between the two daughter cells during mitosis, the nuclear envelope (NE) has to dissolve at the correct moment. The NE comprises a boundary between the cytoplasm and the nucleus and is formed by two membranes, the inner and the outer nuclear membrane, which are separated by the perinuclear space (52, 53). However, NE is not a closed
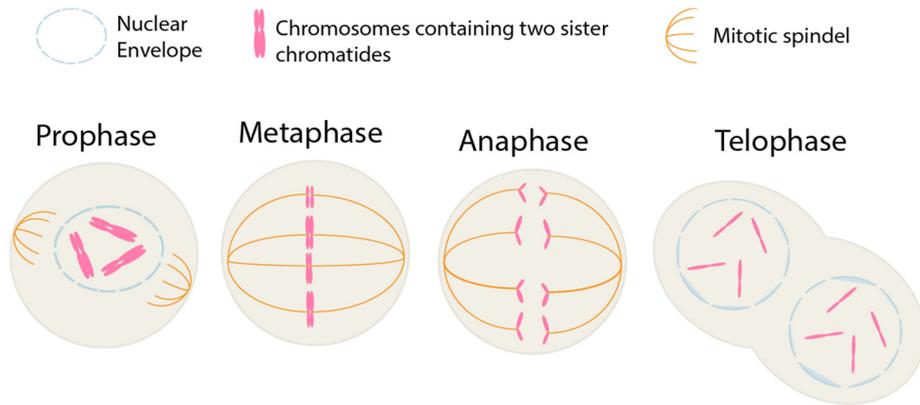
Figure 3. Schematic illustration of the different phases of mitosis.
In prophase, the DNA is condensed and the nuclear membrane starts to dissassemble. During metaphase, the condensed chromosomes are attached to the mitotic spindle and align at the metastatic plane. At anaphase, the centromeres are disjoined and the sister chromatids are segregated by the microtubules. In telophase, the nuclear membrane forms around the sister chromatids and two new daughter cells are formed. (Modified from Malumbres and Barbacid 2009 (54)).

membrane and is perforated by pores, nuclear pore complexes (NPC), where the inner and outer nuclear membranes are fused together. The NPCs function as sieves and allows transport between the cytoplasm and the nucleus (55). The inner nuclear membrane is in contact with both the chromatin and the nuclear lamina (56–58). Both the nuclear membrane and the nuclear lamina is a meshwork of intermediate filaments that provide support to NE (57, 58). The main component of the nuclear lamina is the lamins together with lamina-associated proteins (57). The nuclear lamina plays a crucial role in chromatin organization, DNA replication, cell differentiation and apoptosis. In addition, proper disassembly and reassembly of the nuclear lamina during mitosis is required in the cell division process. The NE is not only an important barrier in the cell, as it is also involved in chromatin organization, nuclear positioning and regulation of gene expression (52).

Disassembly of the nuclear envelope is a multistep process and is controlled by activation of the mitotic kinase, CDK1, which phosphorylates various NE components. First the nucleoporins are dispersed, disrupting the NE permeability barrier. Next microtubules attach to the outer surface of the nuclear membrane and help to pull the NE apart, which clears the chromosomes from the NE. Thereafter the disassembly continues with the depolymerization of the lamina caused by CDK1 phosphorylation (59).

Reassembly of a functional NE around the daughter chromatids occurs at the end of anaphase. It is important that the NE assembles around the entire set of

chromosomes to form a single nucleus. This seems to be achieved by two main processes. Firstly, the chromosomes form a compact cluster during late anaphase. This is accomplished by a combination of shortening of individual chromosomes and bringing the chromosomes in closer proximity to each other. Secondly, proteins of the inner nuclear membrane have high binding affinity for chromatin and are thought to anchor NE components to the chromatin facilitating NE assembly (60, 61).

DNA separation

As mentioned above, during mitosis the chromosomes are segregated equally between the two daughter cells. This process is tightly regulated to avoid missegregation of the DNA, which can give rise to malignancies. Separation of the two sister chromatids is achieved by the interaction of the mitotic spindle with the chromosomes at the kinetochores, a protein structure in the centromeres to which the microtubules can attach (62, 63). In addition, it is important that each kinetochore of the sister chromatids are connected to microtubules from different centrosomes.

During mitosis the dynamic properties of the microtubules change and become more instable. The lifespan of the microtubules changes from several min for an interphase microtubule to 15-30 sec for a mitotic spindle microtubule (64–66). The mitotic microtubules are also shorter and more abundant compared to interphase microtubules. By lengthening and shortening the microtubule probe the environment to eventually connect with the chromosome at the kinetochore (62, 63). When all kinetochores are attached to a microtubule the chromosomes move towards the equator of the cell and align. In anaphase the aligned chromosomes are separated by depolymerization of the microtubule plus ends in combination with a sliding movement of the chromosomes along the microtubules facilitated by kinase motor proteins (67–69).

Due to their important function in chromosome-segregation during mitosis, the microtubule functions during cell division are tightly monitored by the spindle assembly checkpoint. This checkpoint monitors the attachment of the mitotic spindle to the chromosomes and is activated if the chromosomes fail to align, due to for example defects in the mitotic spindle or in the centromeres (70–73). If the damage that activates the spindle assembly checkpoint is irreparable, it causes a prolonged state of cell cycle arrest and eventually apoptosis will be induced (74). Therefore, interfering with microtubule dynamics is a common approach for the treatment of cancer and will be discussed in more detail in the next section of this thesis.

Current treatment

The most common cancer treatment is surgery where the tumor and surrounding tissue are removed. Sometimes surgery is not enough and not all tumors are possible to remove with surgery. In these cases, surgery needs to be combined with other methods such as radiation therapy, immunotherapy or chemotherapy.

In this section of the thesis the focus will be on a group of chemotherapeutics that target tubulins. However, to understand the action of tubulin targeting drugs it is essential to understand the function of tubulins in the cells.

Tubulin superfamily

The tubulins belong to a family of proteins known as the tubulin super family. Tubulins are GTPases, implying that they hydrolyze GTP to GDP to regulate their activity. Today the tubulin superfamily contains several distinct protein families in eukaryotes; including alpha-, beta-, gamma-, epsilon-, delta-, zeta and eta-tubulin (75–77). However, only alpha, beta and gamma-tubulin are ubiquitously expressed. Prokaryotes contain a protein related to tubulin, FtsZ, which replaces alpha-, beta- and gamma-tubulin and is involved in the prokaryotic cell division process (76, 78, 79). The most well studied members of the tubulin superfamily are alpha-, beta- and gamma-tubulin, whose functions will be described in this section. In humans the alpha- and beta- tubulin gene families consist of multiple genes, whereas the gamma-tubulin gene family usually only consists of two genes. Alpha- and beta- tubulin where first discovered when they were found to be the main component of microtubules (76). Until 1989 when gamma-tubulin was discovered, in *Aspergillus nidulans,* alpha- and beta tubulin were thought to be the only tubulins (80). Today, it is well known that gamma tubulin has many different functions in cells.

Microtubules

Microtubules are one out of three major cytoskeleton parts within eukaryotes together with intermediate filaments and actin filaments. The microtubule

functions range from maintaining cell shape, separation of chromosomes in mitosis, intracellular transport, positioning of organelles to motility (81–83). Microtubules are assembled into a hollow cylinder from repeated heterodimers of alpha- and beta-tubulin. Due to their wide range of cellular functions microtubules are a very dynamic cytoskeleton. This is achieved by changes in their assembly and disassembly rates, processes that are referred as microtubule nucleation (82, 83). In a cell microtubule nucleation occurs at specific sites, the microtubule-organizing centers (MTOCs), which anchors the microtubule (83, 84). Different species have different MTOCs but all of them depend on gamma-tubulin for nucleation of microtubules. In animals the primary MTOC is the centrosome.

Microtubule nucleation

Alpha- and beta-tubulin heterodimers form the microtubule and the nucleation of the heterodimers is regulated by gamma-tubulin. During microtubule nucleation, beta-tubulin hydrolyzes the GTP to GDP that remains bound until the microtubule is depolymerized (85). These GTP-binding properties give rise to the dynamic properties of microtubules (85–87).

The microtubule hollow is made up of 13 heterodimers of alpha- and beta-tubulin and microtubule nucleates on gamma-tubulin rich complexes (88). Gamma-tubulin forms two types of complexes that contribute to microtubule nucleation; the small gamma-tubulin complex (γTuSC) and the gamma-tubulin ring complex (γTuRC) (82, 89). γTuSC is a sub complex within the γTuRC and consists of two copies of gamma-tubulin and one copy of each of the gamma-tubulin complex proteins 2 (GCP2) and GCP3 (82, 83, 90). γTuRC is a ring shaped complex made up by the association of several γTuSCs with GCP4, GCP5, GCP6 and mitotic-spindle organizing protein associated with a ring of gamma-tubulin (MOZART) proteins (82, 83, 91). Both γTuSC and γTuRC are able to nucleate microtubules. However, a γTuSC has a much lower nucleation ability compared to the whole γTuRC.

Two models for microtubule nucleation on γTuRC have been proposed, the template model and the protofilament model (75). In the template model several γTuSCs form a ring with a cap of GCP4, GCP5 and GCP6. This complex binds to the microtubule minus end through longitudinal interactions with alpha-tubulin and make up the template for the microtubule (92). In the protofilament model, gamma-tubulin forms a ring shape by longitudinal interactions between gamma-tubulin molecules and lateral interactions with the microtubule (82). Nevertheless, based on the crystal structure of gamma-tubulin, which shows that gamma-tubulin preferably interacts laterally, the accepted model today is the template model (90, 93). However, Kollman et al also found that in the crystal structure of the γTuSC, the gamma-tubulin molecules are positioned to far apart to be able to nucleate microtubules, suggesting that the γTuSC exists in two states; an inactive off state and an active on state (90). The change between the inactive and active states are
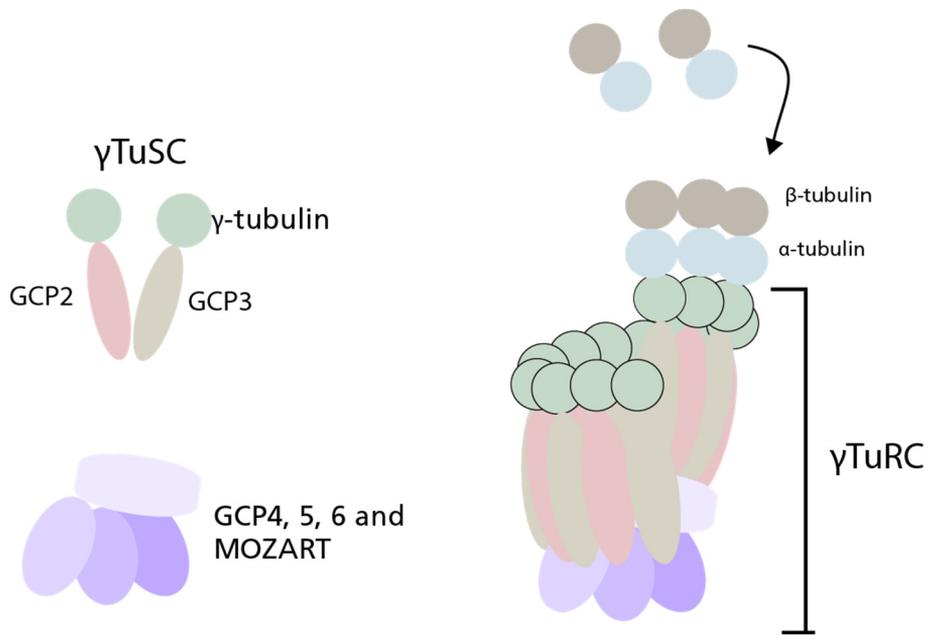
Figure 4. A Simplified illustration of the microtubule nucleation from the template model.
A microtubule nucleates on a the gamma tubulin ring complex (γTuRC). A γTuRC is composed of several small gamma tubulin complexes (γTuSC), which is associated with several different gamma-tubulin associated proteins (GCPs). To form a γTuRC, several γTuSCs interact with one GCP4, 5 and 6. The γTuRC form a template that nucleates the alpha- and beta-tubulin dimers into a hollow microtubule. (Modified from Kollman et al 2011 (82)).

suggested to be regulated by the flexible hinge region of GCP3. Rearrangements of the hinge region causes a conformational change of the γTuSC that brings the two gamma-tubulin molecules closer together leading to the active conformation (83, 90). The active conformation offers a perfect template for the microtubule. However, even though this model is appealing there are still some questions regarding how the MOZART proteins fit in this model. In figure 4 a schematic illustration of the microtubule nucleation according to the template model is presented.

Gamma-tubulin

Apart from nucleating microtubules, during cell cycle, gamma-tubulin regulates the duplication of the centrosome. In interphase, the centrosome is present as a single copy that needs to be duplicated (18). The process of centrosome duplication is controlled by phosphorylation of a specific gamma-tubulin residue, serine 131, by the kinase SADB (17).

So far, all the described gamma-tubulin functions take place in the cytoplasm but gamma-tubulin is also found in the nucleus (94–97). In plant cells gamma-tubulin was found associated with the prekinetochores but also to other sites on the chromosomes during late G2 before transition to mitosis (95). Moreover, Höög et al showed that nuclear gamma-tubulin binds to the E2Fs activators and reduce their transcriptional activity, which affect G1/S transition and S-phase progression (94). Gamma-tubulin has also been found in the nucleoli of mammalian cells associated with C53. C53 is a tumor suppressor protein involved in G_2/M checkpoint activation in response to DNA damage (96). In addition, gamma-tubulin forms a complex with both RAD51 and BRCA1, which are protein involved in DNA damage response (97, 98).

Antimitotic drugs/Microtubule targeting drugs

The microtubule targeting drugs consist of a divers group of substances, many of which are derived from natural compounds. As previously mentioned in this thesis antimitotic drugs act by activating the spindle assembly checkpoint and they can be divided into two principal groups, microtubule stabilizing and microtubule destabilizing substances (99–101). As indicated by the name the microtubule stabilizing substances inhibit depolymerization of the microtubules whereas the microtubule destabilizing drugs prevent microtubule polymerization. Within each of these groups there are different classes of drugs, which are classified based on their binding site on microtubules.

Taxane binding site

One of the first class of microtubule stabilizing drugs discovered was the taxanes. Thus, the most studied binding domain of the microtubule stabilizing drugs is the taxane binding site, which is located in the inner surface of the microtubule lumen on the GDP containing beta-tubulin subunit (101). Upon taxane interaction the conformation of beta-tubulin changes adopting the more stable conformation of the GTP bound beta-tubulin (102). The conformational change shifts the equilibrium of the microtubule dynamic towards polymerization and stabilization. However, the use of this class of drugs is associated with some major limitations including resistance and severe side effects. Therefore, new microtubule stabilizing agents with improved efficacy, like epothilones and laulimalide, has been developed (101, 103–105). Most of these new drugs share similar microtubule stabilizing activities with the taxanes but have unique binding sites.

Vinca domain and colchicine binding site

The microtubule destabilizing drugs affect mainly two binding sites, the vinca domain and the colchicine-binding site (101). The vinca domain contains two distinct binding sites, the vinca site and the peptide site, with similar mechanisms of action. Agents that bind to the vinca site bind near the GTP-binding site on the beta-tubulin subunit and inhibit the GTP hydrolysis, which changes the conformation of the tubulin heterodimer and cause a peeling of the microtubule (106). The peptide binding site overlaps with the vinca binding site on microtubules and interferes with the microtubule dynamics by inhibiting GTP hydrolysis.

Similar to the taxane binding site the colchicine binding site is located on the inner surface of the microtubule lumen but it binds to the interface between the alpha- and beta-tubulin dimer causing a conformational change of the tubulin dimer and this change in conformation suppresses microtubule assembly (101, 107).

Side effects and drug resistance

Due to the importance of microtubules in all cells, microtubule-targeting drugs affect not only malignant cells but also nonmalignant cells in the body. Therefore, one of the main drawbacks with microtubule targeting drugs in cancer therapy are the adverse side effects that are observed, which often affect hematological and neurological processes (99, 100, 108). The neurological adverse effects include peripheral, cranial and autonomic neuropathy with symptoms like constipation, abdominal cramping, motor weakness, numbness, and vocal chord dysfunction (99, 109). The adverse effects of microtubule targeting drugs on neurons are likely due to their interference with the axonal microtubules, which are crucial for transport in neurons. The symptoms associated with hematopoietic adverse effects are mainly neutropenia and is probably associated with the inhibition of microtubules in this rapidly dividing population of cells.

Another problem with the microtubule targeting drugs in the clinical setting is drug resistance, which is currently a topic under much investigation. The most common and efficient mechanisms for a cancer cell to obtain drug resistance is overexpression of drug efflux pumps, which can pump the anti cancer drug from the cell, thereby lowering the drug concentration (103, 108, 110). The overexpression of efflux pump can convey resistance to a large number of structurally and mechanistically different drugs. Other mechanisms that cause resistance are alteration to the microtubules themselves, either by alterations of tubulins, microtubule dynamics or expression of different tubulin isoforms (111). Cancer cells are also known to change their response to checkpoint activation,

induction of apoptosis and to be able to alter their drug metabolism (109, 111). There is still a lot to learn about different mechanisms underlying drug resistance and how cancer cells evade the actions of different drug to be able to develop new and more efficient drugs in the future.

Gamma-tubulin targeting drugs

The agents discussed this far have only targeted the alpha- and beta-tubulin components of the microtubules. In recent years more effort has gone into finding drugs that interact with gamma-tubulin and gamma-tubulin containing complexes but to date there are no approved drugs that target gamma-tubulin function (112, 113). Friesen et al suggest that targeting the nucleation activity of gamma-tubulin in tumors with an overexpression of gamma-tubulin as a possible drug target (113). In addition, they provide evidence for that both colchicine and combretastatin 4-A bind to gamma-tubulin (113). Nevertheless, they emphasized the need for more specific and selective inhibitors as both of these drugs are known to interact with microtubules. In addition, Cala and colleagues showed that the interface between gamma-tubulin and GCP4 contain a binding pocket, which can be targeted to inhibit the gamma-tubulin nucleation of microtubules (112). However, in both of these studies even though the authors aimed to target gamma-tubulin function, the major goal of these studies was to interfere with the microtubule dynamics. Thus, most likely these therapeutic regimes will also suffer from the side effects associated with microtubule targeting drugs.

In contrast, recent evidence suggests that targeting the nuclear activity of gamma-tubulin in a subset of cancer cells will kill cancer cells without affecting microtubule dynamics (3). In this study the authors show that reduced protein levels of gamma-tubulin in tumor cells with a non-functional pRB pathway induce apoptosis by an E2F mediated up-regulation of the protein levels of pro-apoptotic caspase 3 (3). Based on these findings, the authors suggested that inhibition of nuclear gamma-tubulin opens up for a new strategy that target tumor cells without interfering with microtubule dynamics. Thus development of a therapeutic regime that targets the activity of nuclear gamma-tubulin may result in the development of a novel treatment that is better tolerated and give rise to fewer side effects.

Tumor metabolism: an overview

One of the oldest subjects within cancer research is the field of cancer metabolism and the role of the altered metabolic pathway in progression of malignancies. Otto Warburg first studied the field in the beginning of the 20th century. He presented evidence showing that cancer cells upregulate their fermentation of glucose to lactate even in the presence of high oxygen levels, through the process of aerobic glycolysis (114). It was originally thought that the underlying mechanism in aerobic glycolysis was a dysfunctional mitochondria respiration (115, 116). However, recent evidence demonstrates that this is not the case and in fact mitochondria function is crucial for tumor growth (115).

The mitochondrion is an abundant cytoplasmic organelle thought to originate from the fusion of an endosymbiotic prokaryote and a eukaryotic cell (117). The mitochondria are responsible for most of the energy production in the cell, through the production of ATP by oxidative phosphorylation (118, 119). In addition to its role in ATP production mitochondria is involved in several other cellular functions, including regulation of calcium homeostasis, heme synthesis, amino acid synthesis and apoptosis (120).

The mitochondria are composed of two membranes, separated by an intermembrane space, surrounding the mitochondria matrix that contains the mitochondrial DNA (mtDNA) (117, 121). Another structure in the inner membrane is the cristae that give rise to the characteristic appearance of the mitochondria. The cristae are composed of invagination of the inner membrane to increase the surface area to maximize the ATP production (122). Figure 5A show a schematic overview of the mitochondria structure. During the evolution of the mitochondria most of the genes encoding mitochondrial proteins have been moved to the nucleus and are synthetized in the cytoplasm. The mtDNA still contains a circular genome coding for some essential proteins involved in the respiratory complexes, a couple of tRNAs and rRNAs (117). Therefore, mitochondria biogenesis is a complex process, involving import and assembly of components synthesized in the cytoplasm to the mitochondria, and regulating mitochondria homeostasis is essential for maintaining cellular homeostasis (121). However, the exact mechanisms controlling mitochondrial mass and homeostasis are currently not known.
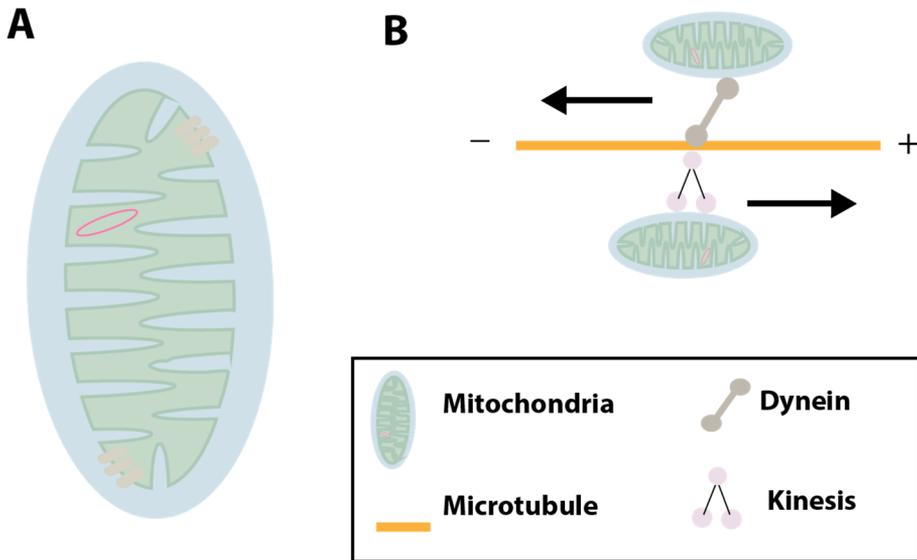
Figure 5. Simplified illustration of the mitochondria structure.
A) The mitochondrion is an organelle composed of a double membrane. The inner membrane contains invaginations that are called cristae, which increase the surface area and energy production. **B)** The mitochondria move along the microtubules with the help of two motor proteins, kinesin and dynein. Dynein move the mitochondria towards the minus end of the microtubules and kinesin towards the plus end. (Modified from Boldogh and Pon 2007 (123)).

The mitochondria form a dynamic meshwork and the number and activity of mitochondria within a cell varies with energy need through the process of fission and fusion (121, 124, 125). In order for a cell to obtain the most out of mitochondria, the cell controls both the activity and positioning of mitochondria. The localization of mitochondria depends on interaction with the cytoskeleton. In higher eukaryotes, mitochondria are known to interact with all three types of cytoskeletal elements, i.e. microtubules, actin filaments and intermediate filaments (123, 126). These interactions are most extensively studied in neurons. The first cytoskeletal component proven to interact with the mitochondria is the microtubules (127, 128). As described previously microtubules are dynamic structures responsible for a variety of cellular functions. The movement of mitochondria by microtubules is dependent on the microtubule-associated motor proteins kinesin and dynein, which is shown in figure 5B (123, 126, 129). Dynein move the mitochondria in the direction of the microtubule minus end, whereas kinesin dependent movement is directed towards the microtubule plus end. The actin filaments and intermediate filaments involvement in mitochondria movement are less well studied. However, it is thought that actin filaments might assist microtubule in mitochondria movement over short distances, through the

assistance of the actin associated motor protein myosin (130). Intermediate filaments is a collection name for more than 50 different proteins that are divided into three main classes, keratins, neurofilaments and vimentin (and vimentin-like filaments), which give mechanical strength to the cell (123, 131). The exact role of intermediate filaments in controlling mitochondria is currently not well studied, but the current view is that they may act as anchorage, which keeps the mitochondria stationary at a specific location (132, 133).

As highlighted in this section many of the molecular components connecting the cytoskeleton and the mitochondria are still unknown, but it is apparent that the cytoskeleton is important for regulation of mitochondria function and location. In the fourth paper of this thesis we have investigated the role gamma-tubulin might have in regulation of mitochondria homeostasis.

The Present Investigation

Aims

The overall aim of the projects presented in this thesis was to investigate the potential of gamma-tubulin as a drug target for the treatment of RB negative tumors and to characterize novel cellular functions of gamma-tubulin.

Specific aims

- To find novel compounds that inhibits the nuclear function of gamma-tubulin.
- To evaluate the role of gamma-tubulin in nuclear assembly.
- To characterize the formation and protein content of gamma-tubulin fibers.
- To study the possible functions of a meshwork form of gamma-tubulin in cellular homeostasis.

Results

Paper I - Therapeutic targeting of nuclear gamma-tubulin in RB1-negative tumors

In this paper we have used three known microtubule targeting agents (paclitaxel, colcemid and citral) to test if the substances specifically inhibited the nuclear activity of gamma-tubulin. Compounds that interfere with the nuclear activity of gamma-tubulin will affect the transcriptional activity of E2F. Cells that transiently expressed a luciferase reporter plasmid containing E2F-binding sites coupled to a luciferase reporter gene were treated with various inhibitors. Expression of the luciferase gene is repressed by the binding of the E2F-gamma-tubulin complex to the E2F-binding sites. Consequently, if a gamma-tubulin inhibitor interferes with the nuclear activity of gamma-tubulin an increased activity of E2F will lead to a higher luciferase activity, which is shown in figure 5. We also assessed the effectiveness and specificity of the various compounds by testing the effect of drugs on microtubule regrowth, on cell cycle and on the death of RB negative cells.
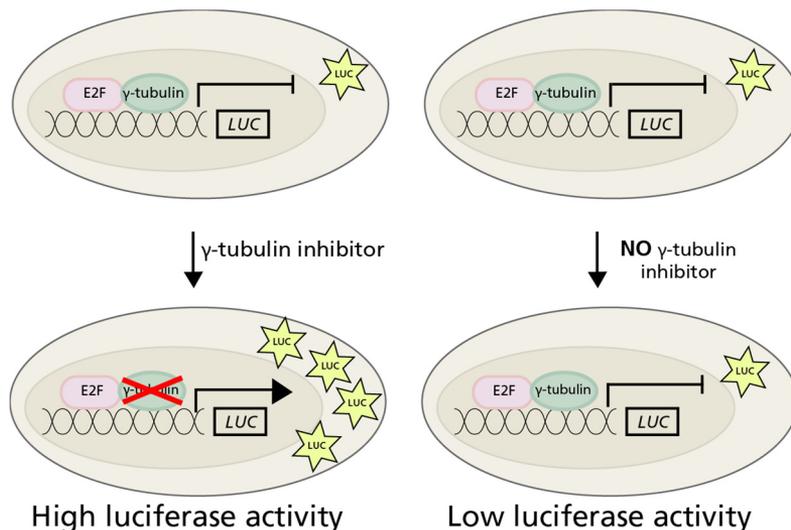
Figure 6. A schematic illustration of the luciferase assay used to screen for gamma-tubulin inhibitors.
A luciferase gene is coupled to a promoter containing E2F binding site and transfected into cells. In untreated cells the luciferase activity is repressed by the E2F-gamma-tubulin complex but when cells are treated with a gamma-tubulin inhibitor E2F become transcriptionally active and the luciferase activity increases. In contrast, there will be no change in the luciferase activity after treatment with a substance that does not inhibit gamma-tubulin. (Adopted from Lindström et al 2015 (134)).

The citral analogue citral dimethyl acetal increases E2F activity without affecting microtubule dynamics

We found that of the three substances tested in the luciferase assay only citral increased the luciferase activity. Citral also increased the protein levels of RB, which suggested that citral inhibited the nuclear function of gamma-tubulin. However, further testing of citral showed that citral affected the microtubule regrowth. To find a more specific gamma-tubulin inhibitor, we examined several analogues to citral and found that citral dimethyl acetal (CDA) and citronellal also increased the transcriptional activity of E2F. Notwithstanding, citronellal affected the microtubule outgrowth, whereas CDA affected neither microtubule depolymerization nor growth.

The cytotoxic effect of CDA is E2F mediated and is dependent on gamma-tubulin levels and the pRB-status in cells

To further characterize the effect of CDA on the RB/gamma-tubulin pathway, we continued to examine how CDA affected several cell lines with various defects in the pRB-signaling pathway by flow-cytometry and western blot analysis. We found that cells reacted in a dose dependent manner to CDA treatment depending on the pRB status in the cell. Cells lacking pRB expression were more sensitive to CDA treatment than cells with a functional pRB-signaling pathway. Moreover, as expected of a gamma-tubulin inhibitor, CDA increased the protein levels of RB in several cell lines (3). We then created several stable cell lines with different expression levels of RB, gamma-tubulin and E2F1. Reduction of RB1 or gamma-tubulin, by shRNA mediated knockdown, sensitized cells to CDA treatment. However, overexpression of gamma tubulin or expression of RB1 in cell lines normally lacking RB expression reduced the cytotoxic effect of CDA. Finally reduced levels of E2F1 attenuated the effect of CDA treatment demonstrating that the cytotoxicity caused by CDA was E2F mediated.

CDA binding to gamma-tubulin is mediated by binding to Cys13

Here next, we used a mutational analysis and a ligand-based chemoinformatic approach to characterize the CDA binding-site on gamma-tubulin. CDA is set-up for a Michael addition and thus may bind covalently to a cysteine residue (135). To find the CDA binding pocket at the surface of gamma-tubulin, we used a computational method and the key binding-site found at the surface of gamma-tubulin was the GTP/GDP binding cavity. Sequence alignment between alpha- beta- and gamma-tubulin lead us to cysteine 13 (Cys13). When Cys13 was mutated to an alanine in gamma-tubulin and the protein was stably expressed in cell lines, we found that the deadly effect of CDA treatment on cells was attenuated, demonstrating that CDA binding to gamma-tubulin is Cys13 mediated. Finally, a chemoinformatic model using two drug databases containing FDA-

approved compounds led us to another compound that was predicted to bind Cys13, dimethyl fumarate (DMF). DMF is currently used for treatment of multiple sclerosis and psoriasis. We therefore tested DMF's effect on two cell lines with different RB status and found that, as CDA, DMF treatment caused cell death in a pRB-dependent manner. Taken together these results indicate that interference with the GTPase domain of gamma-tubulin has cytotoxic effects in tumors with an impaired RB signaling pathway.

CDA reduce tumor growth in vivo

Finally, to study the *in vivo* effect of CDA treatment in tumor growth, we xenografted mice with two cell lines, one pRB-deficient and one stably expressing pRB1. The tumors formed were heterogeneous and grew very rapidly so the mice had to be sacrificed before the treatment were significant. However, we could see a trend towards reduced tumor growth in both tumor types. We found this somewhat surprising and decided to study the pRB levels and the phosphorylation status of pRB in the two tumor types. We found that the pRB1 positive tumors had a phosphorylated pRB and thus pRB was inactivated, providing us with an explanation to why both tumor types reacted similar to CDA treatment. We then preformed another animal experiment were we injected the tumor cells together with matrigel to obtain a more homogenous tumor-growth and in this model CDA treatment significantly reduced tumor growth.

In short, this paper shows that CDA binds to the GTPase domain of gamma-tubulin and interacts with Cys13. By targeting gamma-tubulin's nuclear function it is possible to cause cell death in cell lines with a dysfunctional pRB-signaling pathway. In addition, CDA treatment reduced xenograft tumor growth in mice. Taken together these results show that it is possible to develop drugs that target the nuclear activity of gamma-tubulin without affecting microtubule dynamics and our work paves the way for the development of safer drugs with fewer side effects.

Paper II - Gamma-tubulin coordinates nuclear envelope assembly around chromatin

At the end of cell division, formation of the NE around the chromatin of the two daughter cells is crucial for the maintenance of genomic integrity. However, the mechanisms underlaying the NE formation are currently not well understood. Microtubules has been implicated to play a role during nuclear formation (136) but the role of nuclear gamma-tubulin have not yet been elucidated. The aim of this paper was to investigate if gamma-tubulin is involved in nuclear formation.

Gamma-tubulin is associated to the chromatin and forms a boundary around DNA during nuclear formation

To investigate gamma-tubulins functions during nuclear assembly, we studied the formation of the nucleus in *Xenopus laevis* egg extracts. We choose to use this system as it allowed a more efficient depletion of gamma-tubulin compared to knockdown experiments in cell lines. Immunofluorescence staining of xenopus sperm revealed that gamma-tubulin was found localized throughout the DNA whereas alpha- and beta-tubulin were only found in the centrosomal end of the sperm.

During nuclear formation we observed that the sperm underwent four distinct morphological stages during nuclear assembly. During the first stage the nucleus was condensed and became decondensed during the second stage. In the third stage the nucleus was almost formed and in the last stage it grew in size. In the first three stages gamma-tubulin was found bound to the DNA and in the final stage gamma-tubulin became enriched at the NE. By treating the extracts with colcemid we excluded the involvement of alpha- and beta-tubulin in nuclear formation. We also saw that the localization of gamma-tubulin during nuclear formation was not affected in the presence of colcemid, which suggested that gamma-tubulin performed microtubule independent functions during nuclear assembly.

Chromatin-bound gamma-tubulin recruits the lamins to the chromatin

To investigate if gamma-tubulin was necessary for nuclear assembly we performed several depletion and add back experiments in *Xenopus laevis* egg extracts. We found that depletion of gamma-tubulin in both sperm and egg extract significantly decreased nuclear assembly and add back of gamma-tubulin rescued the nuclear formation, which proved that gamma-tubulin is necessary for nuclear formation. We saw that gamma-tubulin formed a boundary around the chromatin before the recruitment of the lamina and nuclear membranes, indicating an involvement of gamma-tubulin in recruitment of the lamins and nuclear membranes. Moreover in the absence of gamma-tubulin neither the lamina nor the nuclear membrane were formed, but the addition of recombinant gamma-tubulin restored the NE formation. To elucidate which domain of gamma-tubulin that was involved in nuclear formation, we tested the ability of various recombinant gamma-tubulin mutants to induce nuclear formation in gamma-tubulin depleted egg extracts. We found that only full-length and C-terminal DNA-binding region of gamma-tubulin bound to chromatin and reversed the effect of gamma-tubulin depletion. To examine if the defects observed when interfering with gamma-tubulin were due to impaired lamina formation we added recombinant lamin B3 to gamma-tubulin depleted sperm and monitored nuclear formation. In the absence of gamma-tubulin lamin B3 was not recruited to the chromatin and neither lamina

nor NE were formed, providing further evidence that lamins are recruited to the chromatin by chromatin-bound gamma-tubulin.

Gamma-tubulin forms a meshwork of strings in cells

As gamma-tubulin localizes to both the cytoplasm and the nuclear compartment in cells, we examined how the two pools were connected. We found that similar to lamins, gamma-tubulin formed a meshwork of strings on the NE that were intertwined with lamin B. In contrast to the lamin B meshwork that only is present on the nuclear boundary, the gamma-strings were found throughout the nucleus. To exclude the possibility that the observed gamma-strings were fixation artifacts, we used two immunoelectron microscopy (EM) methods to elucidate the structure of the gamma-string meshwork. Both methods showed that gamma-tubulin formed strings in the cytoplasm, the nuclear membrane and the nucleus. Analysis of gamma-tubulin immunoprecipitates from biochemically fractionated cells enclosed all three components of the lamina in the chromatin fraction, whereas only lamin B was associated with gamma-tubulin in the cytoplasmic and nuclear membrane fractions, which further strengthened our belief that lamin B was recruited to the NE by gamma-tubulin.

Both the gamma-tubulin N- and C-terminal associate with lamins but the C-terminal is required for binding of chromatin

We also analyzed time-laps images of mitotic cells to investigate the connection between gamma-tubulin and lamina formation during nuclear assembly. We observed that gamma-tubulin formed a boundary around the mitotic chromosomes that was sustained in the daughter cell. In contrast, the lamina remained in the cytoplasm until late mitosis when the lamina assembled at the gamma-tubulin boundary. Finally we wanted to examine the role of the gamma-tubulin N- and C-terminal regions in nuclear formation. We found that both the gamma-tubulin N- and C-terminal associated with lamins and cells could form a lamina. However, only the gamma-tubulin N-terminal formed a lamina in the absence of chromatin.

In short, in this paper we show that gamma-tubulin forms a network of gamma-strings in cells, which connect the nuclear and cytoplasmic compartments. We also show that gamma-tubulin associates with the chromatin and during nuclear formation gamma-tubulin forms a boundary around chromatin, which is necessary for recruitment of lamina and NE components. We also provide evidence that the C-terminal region of gamma-tubulin helps to assure that nuclear assembly occurs around chromatin. The mechanism controlling the formation of the gamma-string meshwork still needs further evaluation. However, *in vitro* recombinant gamma-tubulin spontaneously forms a meshwork of strings. Moreover, previous work has shown that the C-terminal region of gamma-tubulin forms tubule like structures in cells, suggesting that the C-terminal region might be involved in formation of

gamma-strings (137). Altogether our results indicate that the gamma-string meshwork is involved in coordinating nuclear and cytosolic events during cell division.

Paper III - Characterization of gamma-tubulin filaments in mammalian cells

It was originally proposed that gamma-tubulin forms filaments as both alpha- and beta-tubulin and the gamma-tubulin analog in bacteria, FtsZ, form filaments. In addition, in the monkey kidney cell line Cos, overexpression of gamma-tubulin formed filaments called gamma-tubules. The aim of this paper was to study the ability of gamma-tubulin to form filaments in cells.

Gamma-tubulin form filaments in cells
We investigated the ability of gamma-tubulin to form filaments, henceforth named gamma-tubules, by immunofluorescence staining. We saw that gamma-tubulin formed gamma-tubules in cells but that the detection was dependent on fixation method. The method that best preserved gamma-tubules was when the cells were first fixed for 3 minutes in 4% paraformaldehyde followed by a 3 minute permeabilization step with methanol/acetone. The gamma-tubules were found both in the cytoplasm and close to the NE in approximately 20% of cells and the gamma-tubules' length varied. To prove that the gamma-tubulin immunostaining of the gamma-tubules was specific, we knocked down the expression from *TUBG1* and *TUBG2* genes by using single guide RNA (sgRNA), which abolished the immunofluorescence staining. Moreover, gamma-tubules were not ubiquitinated, which excluded the possibility that the gamma-tubules were protein aggregates. To further prove that the gamma-tubules were not the result of fixation artifacts, we imaged high-pressure frozen cells with immunoelectron microscopy using a gamma-tubulin antibody. We saw that the gamma-tubulin antibody recognized filaments in the cytoplasm that were between 20 to 25 nM in diameter, further supporting that gamma-tubules form cellular structures.

Gamma-tubulin forms a cellular cytoskeleton that is intertwined with microtubules
Here next, we investigated whether gamma-tubules were found in live cells. Cells stably expressing a GFP-tagged gamma-tubulin showed that the GFP-tag impeded the incorporation of the GFP gamma-tubulin to gamma-tubules. We hypothesized that gamma-tubules are a component of the gamma-tubulin meshwork. We analyzed immunofluorescence images and found that the gamma-strings were attached to both the centrosomes and the gamma-tubules and formed a single cellular skeleton. Finally, we examined the interactions of gamma-tubules and gamma-strings with other cytoskeletal elements and found that gamma-tubulin

colocalized with vimentin, lamin B, actin and microtubules. Also, when we compared the spatial three-dimensional location of gamma-tubulin with that of microtubules we found that they formed two distinct but intertwined meshworks.

Gamma-tubules are globular units formed by repeated γTuRCs

To investigate the gamma-tubule structure we used structured illumination microscopy, which showed that the tubules were made up of globular units. As cytosolic gamma-tubulin forms two types of complexes; the γTuSC and the γTuRC, we studied the content of GCPs in gamma-tubules by immunofluorescence staining. We saw that GCP2, GCP3 and GCP6 associated with gamma-tubules, which indicate that the gamma-tubules are formed by repeated γTuRCs.

Formation of gamma-tubules are microtubule independent and occurred at gamma-tubulin foci

To investigate if the gamma-tubules are polar structures and their interactions with actin and microtubules we interfered with these structures using several different treatments. We found that treatment with cold and colcemid, a microtubule interfering agent, reduced the number of gamma-tubules, which suggested that gamma-tubule formation either was microtubule dependent or dependent on the gamma-tubulin colcemid-binding domain. We also found that interfering with the gamma-tubulin GTPase domain reduced the number of gamma-tubules found in cells without affecting the microtubules, which indicated that the GTPase domain was important for gamma-tubule formation. We also observed that reassembly of the gamma-tubules in the cytoplasm occurred at gamma-tubulin foci, in comparison to the microtubules that were nucleated at the centrosomes. Furthermore, treatment of cells with low concentrations of colcemid led to the formation of short gamma-tubules, but impeded microtubule regrowth, which demonstrated that gamma-tubule formation was microtubule independent.

In summary, in this paper we show that endogenous gamma-tubulin forms cytosolic tubular structures close to the NE and that in the absence of mitotic microtubules the gamma-tubules were present at condensed chromosomes. We also demonstrate that the gamma-tubules were formed at gamma-tubulin foci and contained GCP2, GCP3 and GCP6. In addition, the gamma-tubules together with centrosomes and the gamma-strings form a cellular meshwork that connects the cytosolic with the nuclear compartment. Finally we proved that it was the gamma-tubulin GTPase domain that was necessary for the gamma-tubule formation and together these findings provide insights into the gamma-tubulin functions and dynamics.

Paper IV - the GTPase domain of gamma-tubulin controls mitochondria homeostasis

In this paper we show that gamma-strings can assemble into tubular-like structures, gamma-tubules, which grow from the nuclear envelope towards the plasma membrane. We investigated the interactions of the gamma-tubulin meshwork with other cytoskeletal elements and immunofluorescence staining showed that the gamma-string meshwork colocalized with actin filaments, microtubules and the intermediate filament vimentin throughout the cytoplasm. In Furthermore, gamma-tubules were localized in close proximity to the nucleus where other cytoskeletal components are spare. However, when we interfered with these cellular cytoskeletons, the gamma-tubulin meshwork was not affected. This suggested that gamma-tubulin forms an independent meshwork. We also monitored the gamma-tubulin meshwork after treatment with gamma-tubulin inhibitors, CDA and DMF, and observed a reduction in the number and in the length of the gamma-tubules that were formed. Since CDA and DMF affect the GTPase domain of gamma-tubulin, this suggested to us that the gamma-tubulin GTPase domain was involved in controlling the dynamics of the gamma-tubulin meshwork.

Gamma-tubulin associates with mitochondria and shape the mitochondria meshwork in a GTPase-dependent manner

As the gamma-tubulin meshwork formed by the C-terminal region of gamma-tubulin (137) resembles that formed by mitochondria close to the nucleus we investigated the colocalization between gamma-tubulin and the mitochondria network. We observed that the gamma-tubulin meshwork colocalized with several mitochondria markers. As mentioned previously mitochondria stem from a fusion of an endosymbiotic bacteria with a eukaryotic cell and lack the gamma-tubulin homologue FtsZ (78, 138). Therefore, we investigated if FtsZ function is replaced by gamma-tubulin in mitochondria. We showed that the gamma-string meshwork connected the mitochondria with each other and with the nucleus and that gamma-tubulin was present both inside and outside of the mitochondria. Taken together, this indicates that gamma-tubulin forms a network that anchors the mitochondria within the cell. We also observed that sh-RNA induced reduction of gamma-tubulin disrupted the mitochondrial meshwork and this effect was reversed by expression of shRNA-resistant gamma-tubulin but not by gamma-tubulin with a mutated GTPase domain. This provides evidence that gamma-tubulins GTPase domain is necessary for organization of the mitochondrial meshwork.

Gamma-tubulin control mitochondrial activity

As gamma-tubulin is known to bind to chromatin we wanted to examine whether gamma-tubulin also associated with the mtDNA. We performed a chromatin

immunoprecipitation assay with gamma-tubulin antibodies and found that gamma-tubulin is present on the mtDNA and that the levels of gamma-tubulin regulate the expression of MTCO2. We examined the effect of the gamma-tubulin meshwork on mitochondrial respiration with a Seahorse assay, which measured the cellular oxygen consumption rate (OCR), in cell lines with different gamma-tubulin protein levels. Contrary to what we expected only CDA treatment significantly reduced the OCR in cells. To examine if the contradictory findings were due to an adaptation of the number of mitochondria in the cell lines with impaired function or reduced levels of gamma-tubulin we measured the ratio between mtDNA and nuclear DNA by quantitative PCR and found an upregulation of the mitochondrial mass. This explained the opposing effects we saw in the Seahorse assay. CDA did not cause any change in mitochondrial mass, which was probably due to the short treatment time. The adaptation of the mitochondria's respiratory capacity occurs rapidly in response to variations of gamma-tubulin levels.

Interfering with the GTPase domain of gamma-tubulin affects mitochondria organization

Finally we proposed that cells use the gamma-tubulin meshwork to connect the activity of the mitochondria with the metabolic state of the cell and to test this we treated cells with the gamma-tubulin inhibitor DMF. We observed that DMF impaired both the mitochondria respiration in a WST-1 assay and disrupted both the mitochondria and the gamma-tubulin meshwork. As both treatment with CDA and DMF, which interfere with the GTPase domain of gamma-tubulin, alter the mitochondria meshwork we suggested that the GTPase domain in gamma-tubulin is necessary for mitochondria organization.

In short, this paper shows that gamma-tubulin forms a string meshwork that interacts with mitochondria. Disruption of the gamma-string meshwork, either by interfering with the gamma-tubulin protein levels or the GTPase function, affects mitochondria respiration and expression of mitochondria related genes. Therefore we propose that gamma-tubulin forms a network that provides organization to mitochondria and controls mitochondria homeostasis through the involvement of the gamma-tubulin GTPase domain. However, the exact mechanism by which hydrolysis of GTP would facilitate this process needs further investigation.

Conclusion

In the papers on which this thesis is based, we have characterized novel functions of gamma-tubulin and investigated the potential of gamma-tubulin as a chemotherapeutic target in the treatment of cancer.

We conclude that:

- Inhibition of the nuclear activity of gamma-tubulin by CDA causes cell death in cells with a dysfunctional pRB-singnaling pathway.

- Gamma-tubulin forms a protein meshwork in cells that is necessary for lamina formation during nuclear assembly.

- Gamma-tubule formation is dependent on the gamma-tubulin GTPase domain and the gamma-tubules form a cytoskeletal network that connects the cytoplasm with the nuclear compartment.

- The gamma-tubulin meshwork associates with the mitochondria and controls mitochondria homeostasis and organization.

Populärvetenskaplig sammanfattning

Idag sker behandling av cancer med läkemedel som framkallar svåra och jobbiga biverkningar och behovet av nya och bättre läkemedel är stort. Biverkningar som uppstår på grund av dagens läkemedel beror till stor del på vilka celler som påverkas vid behandlingen. Med dagens läkemedel dödas alla celler i kroppen som delar sig och inte bara cancercellerna. Anledningen till att det är de celler som delar sig man vill komma åt beror på att cancer uppstår eftersom vissa celler i kroppen börjar dela sig okontrollerat.

Normalt är en cell beroende av signaler från omgivningen som talar om att den ska börja dela sig men en cancercell kan dela sig utan dessa signaler. För att en cancercell ska kunna dela sig obegränsat måste den ändra gener som styr cellcykeln, den process som cellen genomgår för att kopiera sin arvsmassa och dela sig i två identiska dotterceller. I en normal cell finns det dessutom ytterligare kontroller som kan stoppa celler från att dela sig vid fel tillfälle. Även dessa processer är ofta förändrade i cancerceller eftersom de annars hindrar deras framfart.

Många proteiner behövs under cellens delningsprocess och i denna avhandling har jag fokuserat på två av dessa, retinoblastoma och gamma-tubulin. Om en normal cell inte får rätt signaler från omgivningen stoppar retinoblastoma cellen från att fortsätta i cellcykeln och cellen kan inte dela sig. Man kan alltså säga att retinoblastoma fungerar som en portvakt i första delen av cellcykeln och ser till att bara de celler som är redo får dela sig. Det är därför vanligt att retinoblastoma är avstängd i många olika typer av tumörer.

För att cellen ska kunna dela sig måste dessutom arvsmassan delas upp mellan de två dottercellerna och det är viktigt att båda cellerna får en hel uppsättning av arvsmassan. För att dela upp arvsmassan bildar cellen ett nätverk av proteiner som påminner om ett spindelnät. Nätverket fäster i arvsmassan och drar isär det till de båda nya cellerna. Gamma-tubulin är det protein som ser till att detta nätverk bildas vid rätt tid och på rätt plats. Om nätverket inte bildas på rätt vis finns det en risk att arvsmassan inte delas lika mellan dottercellerna.

Eftersom många cancerläkemedel idag är riktade mot att förhindra bildandet av nätverket som delar upp arvsmassan mellan dottercellerna påverkas inte bara cancercellerna utan även alla andra celler i kroppen som delar sig. I vår

forskningsgrupp har man tidigare visat att om man sänker nivåerna av gamma-tubulin i cancerceller med ett icke fungerande retinoblastomaprotein så dör dessa celler. Om man däremot sänker nivåerna av gamma-tubulin i celler som har ett fungerande retinoblastomaprotein så överlever dessa celler. Eftersom alla kroppens friska celler har ett fungerande retinoblastoma protein kan gamma-tubulin tänkas vara ett nytt mål för cancerläkemedel med färre biverkningar.

I delarbete I har vi undersökt en potentiell gamma-tubulinhämmare och upptäckt att denna substans kan döda celler som saknar retinoblastoma vid koncentrationer där celler med retinoblastoma överlever. Vi har också testat att behandla tumörer med vår substans och då såg vi att tumörerna växte långsammare. När man ska utveckla läkemedel mot nya mål i cellen är det viktigt att man vet så mycket så möjligt om det tänkta målet. Därför ägnade vi delarbete II-IV till att undersöka några hitintills okända funktioner som gamma-tubulin har i cellen.

I en cell sker hela tiden många olika processer, allt från energiproduktion till uttryck av gener i kärnan, som måste kopplas ihop. Detta sker genom att olika nätverk länkar samman olika delar av cellen med varandra. Vi har sett att gamma-tubulin kan bilda två olika nätverk i närheten av kärnan men vi vet inte vad detta nätverk har för funktion. I delarbete II undersökte vi det ena nätverket och såg att det består av tunnare fiber som ser ut som trådar. Vi upptäckte att dessa trådar bildar ett nätverk som binder samman olika delar av cellen med varandra och att det hjälper till att bilda membranet runt cellkärnan efter att cellerna har delat sig. Det andra nätverket som gamma-tubulin bildade var uppbyggt av tjockare fibrer som sträckte sig över kärnmembranet.

I delarbete III undersökte vi vilka andra byggstenar som fanns i gamma-tubulinfibrerna och hur de bildas. Vi såg att en särskild del av gamma-tubulin var tvungen att finnas för att fibrerna skulle bildas. Denna del kallas GTPas-domänen eftersom den binder till en molekyl som kallas GTP. Vi såg också att det krävdes att gamma-tubulin interagerade med flera andra proteiner för att fibrerna skulle bildas. En annan viktig byggsten i cellen som kräver mycket organisation för att fungera korrekt är cellens energifabrik, mitokondrien. Därför undersökte vi i delarbete IV om gamma-tubulin nätverket och mitokondrien var sammanlänkade och om gamma-tubulin kontrollerade mitokondriens funktion. Vi såg att gamma-tubulin samspelar med mitokondrier och organiserar och kontrollerar deras aktivitet. Även detta samspel är beroende av GTPas-domänen i gamma-tubulin.

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