Jens Lüders *Editor*

The Microtubule Cytoskeleton

Organisation, Function and Role in Disease

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Preface

The eukaryotic microtubule cytoskeleton is involved in a large number of essential processes such as cell migration, polarization, and morphogenesis, as well as intracellular transport, positioning of organelles, and segregation of chromosomes during cell division. Fundamental to the function of the microtubule network is the arrangement of microtubules into highly ordered arrays. The assembly and regulation of these arrays and their remodeling during cell cycle progression or cell differentiation are highly complex processes that we only begin to understand. Increasing our knowledge in this area is an important task, since defects in the microtubule network have been implicated in a large number of pathological conditions ranging from malformations during development to degenerative disorders.

This book does not attempt to cover all aspects of this immense complexity, but instead provides an overview on the organization and function of the microtubule cytoskeleton and how its impairment is linked to disease. By focusing on the key mechanisms and by presenting concepts alongside detailed molecular information, the book should be of interest not only to experts but also to nonexpert readers.

In several chapters leaders in the field describe how microtubules are organized at different cell cycle stages and in different cell types; present insight into the structure and function of the centrosome, the main microtubule organizing center; and highlight important proteins and protein complexes that generate and organize microtubules, modulate their properties, and mediate their function. The chapters also contain information on how malfunction of specific components of the microtubule network, caused by genetic mutation or other mechanisms, leads to pathological conditions.

I am grateful to all authors for their excellent contributions and efforts to align these with the scope of this book. Hopefully, by stimulating discussion and research, their work will make a contribution toward a better understanding of the cell and pathobiology of the microtubule cytoskeleton.

Barcelona, Spain
November 2015

Jens Lüders

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Microtubule Organization in Mitotic Cells

1

Sylvain Meunier and Isabelle Vernos

Abstract

Mitosis, the process by which one cell divides into two genetically identical daughter cells, is the most basic process for the development and proliferation of living organisms. In eukaryotes, mitosis involves the transient organization of a sophisticated molecular machine, the bipolar spindle that orchestrates the segregation of the genetic material to the daughter cells. The spindle is a microtubule (MT)-based apparatus whose assembly and function rely on the fine modulation of MT intrinsic dynamic properties and on their spatial and temporal organization. In this chapter, we will focus on the mechanisms of spindle assembly and dynamics. We will discuss some current questions in the field and review the consequences of defective MT function in mitotic cells for human health.

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1.1 Introduction

Almost 200 years ago, Schwann's theory stated that all living things are composed of cells. Later in 1857, Rudolf Virchow postulated that "Omnis cellula e cellula," the generation of new cells from a pre-existing cell, involves a specific process called mitosis.

After the very first division of the fertilized egg, a complex developmental program involving billions of cell divisions takes place to generate the tissues and organs that constitute a full organism. Cell division is also essential in the adult organism. It occurs permanently throughout the life of a human being, playing an essential role in the maintenance and renewal of its tissues and organs.

Flemming in the late nineteenth century described the two main elements in animal cells undergoing mitosis, called "thin filaments" and "chromo elements" (Paweletz 2001): these are microtubules (MTs) and chromosomes. Almost 150 years after this initial description, we have now attained a reasonably good understanding of the mechanism that underlies cell division. In this chapter, we will focus on the general principles underlying spindle assembly and function from an MT centric perspective. For simplicity, we will focus on mitosis in higher eukaryotes, but it is worth keeping in mind that many of the pathways and mechanisms are similar in meiosis, a gamete-specific cell division process.

1.2 Microtubule Basic Properties and Mitosis

Cell division involves the full reorganization of the interphase MT network to assemble the mitotic spindle, a dynamic molecular machine that provides the forces and support for chromosome segregation (Inoue and Sato 1967). To address the mechanism leading to the assembly of the spindle, it is therefore essential to start by revising some essential MT properties.

1.2.1 MT Basics

MTs are hollow tubes of 25 nm in diameter formed by lateral interactions of 13 protofilaments. Each protofilament in turn is formed by head to tail interactions of α-/β-tubulin heterodimers, two closely related tubulin isoforms that bind GTP. This molecular organization defines MT polarity: only α-tubulin subunits are exposed at one extremity called the minus end and only β-tubulin subunits are exposed at the other extremity called the plus end (Fig. 1.1). The resulting polarity along the MT lattice is read by molecular motors that move directionally along the filament either toward the plus or the minus end.

In vitro MTs form spontaneously above a certain concentration threshold of α-/β-tubulin heterodimers and exhibit dynamic properties. In the presence of GTP, MTs grow and shrink, stochastically alternating between these two phases by undergoing catastrophes when switching from the growing state to the shrinking

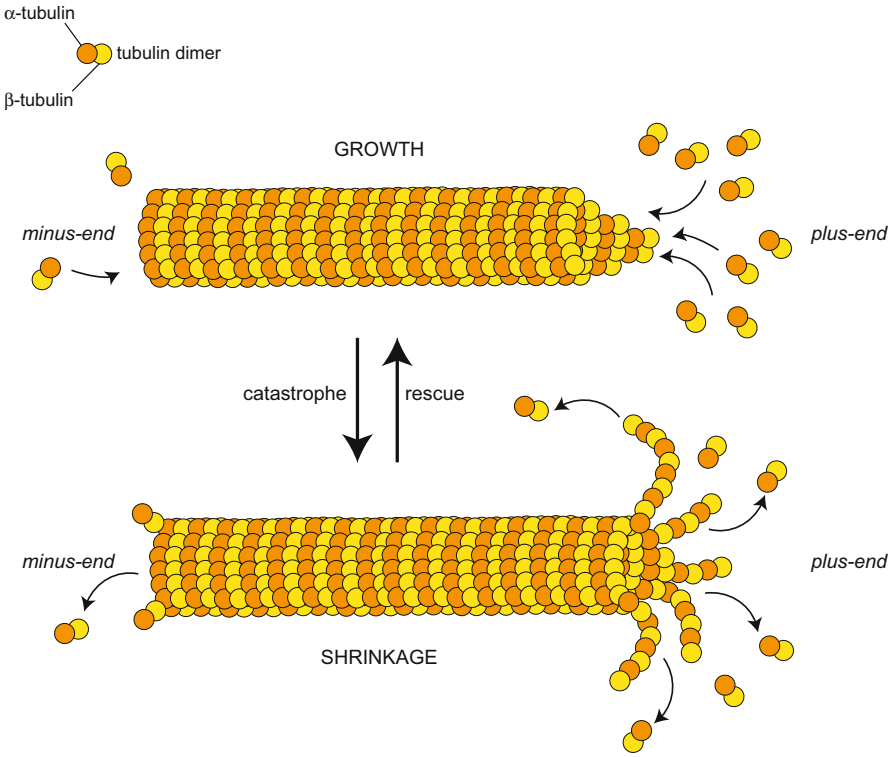

Fig. 1.1 MT basics. Microtubules (MTs) are composed by tubulin heterodimers of α- and β-tubulin. This composition defines a polarity to the MTs; α-tubulin is exposed at the minus end and β-tubulin at the plus end. They are intrinsically dynamic and undergo phase of growth and shrinkage. The switch from a growth state to a shrinking state is called catastrophe, and the opposite switch is the MT rescue

state and rescues for the opposite switch (Wade 2009) (Fig. 1.1). These unique properties define MT dynamic instability as named by Mitchison and Kirschner when they first described it in 1984 (Mitchison and Kirschner 1984). Interestingly, they demonstrated that the dynamic properties and average length of any MT population could be described using four parameters: the velocities of growth and shrinkage, and the catastrophe and rescue frequencies (Mitchison and Kirschner 1984). All these events occur at the MT plus ends, while MT minus ends are more stable. MT polarity therefore is also related to the differential dynamic behavior of the two MT extremities.

MT dynamic properties are intimately related to GTP hydrolysis. Indeed, MTs formed with a slowly hydrolysable GTP analogue GMCPP are remarkably stable in vitro. GTP hydrolysis occurs only at the β-tubulin subunit after incorporation of the α-/β-tubulin heterodimer into the growing MT plus end. MTs are therefore mainly composed by GDP-tubulin with a so-called GTP cap at their plus ends. By promoting a change in conformation of the tubulin dimer, GTP hydrolysis generates

a tension within the polymer. When GTP hydrolysis occurs faster than the rate of tubulin incorporation at the plus end, this tension is released by fast depolymerization (Alushin et al. 2014).

MTs therefore have two essential intrinsic properties: they are polarized and they show dynamic instability. These properties are key for most of the events leading to the assembly of the mitotic spindle.

1.2.2 Microtubule Organization in Mitosis

By regulating MT dynamic properties, the cell controls the organization of its MT network. This is key for mitosis when in a timely manner the interphase MT network disassembles to build the mitotic spindle, the essential machinery to segregate the chromosomes. This reorganization is in fact dynamic and occurs through an ordered sequence of phases (Fig. 1.2).

In prophase, the interphase MT network disassembles and the duplicated centrosomes separate to opposite sides of the nucleus along the nuclear envelope. As the centrosome MT nucleation activity increases, they form two asters of highly dynamic MTs. The chromosomes condense inside the nucleus. Prometaphase starts after nuclear envelope breakdown. MTs establish connections with the chromosomes and some of them get stabilized at specific sites on the centromeres, the kinetochores. As these interactions get established, MTs start to organize into a spindle-shaped apparatus (Fig. 1.2). Metaphase is characterized by a mature bipolar spindle in which MT minus ends are focused at the two spindle poles and MT plus ends interdigitate at the center where chromosomes are aligned on the metaphase plate. Once all the chromosomes are correctly attached to both spindle poles, anaphase A starts, and the sister chromatids are pulled apart toward the two opposite poles. In anaphase B, a novel MT-based antiparallel array, the central spindle, assembles in between the separated chromosome masses and promotes their further separation by antiparallel MT sliding. In telophase, two nuclei form and the daughter cells separate completely through cytokinesis and abscission before establishing their interphase MT network (Fig. 1.2).

All the major events driving cell division rely on timely and spatially controlled changes in MT dynamic properties and organization. Based on the MT dynamic instability property, Kirschner and Mitchison (1986) proposed the “search and capture” model for spindle assembly. It postulated that highly dynamic MTs emanating from the separated centrosomes grow and shrink, exploring the cytoplasm until some of them get captured and stabilized by the kinetochores. Since MTs emanate from two centrosomes and each chromosome has two kinetochores, this stochastic process should naturally drive bipolar spindle assembly. Although we know now that the mechanism driving spindle assembly is more complex, the basic principle of this model is still valid: a large number of dynamic MTs are required to efficiently explore the cellular space and attach the chromosomes. However, these MTs may or may not be generated by the centrosomes. In fact, centrosomal MTs alone do not form a functional bipolar spindle (Gruss et al. 2002)

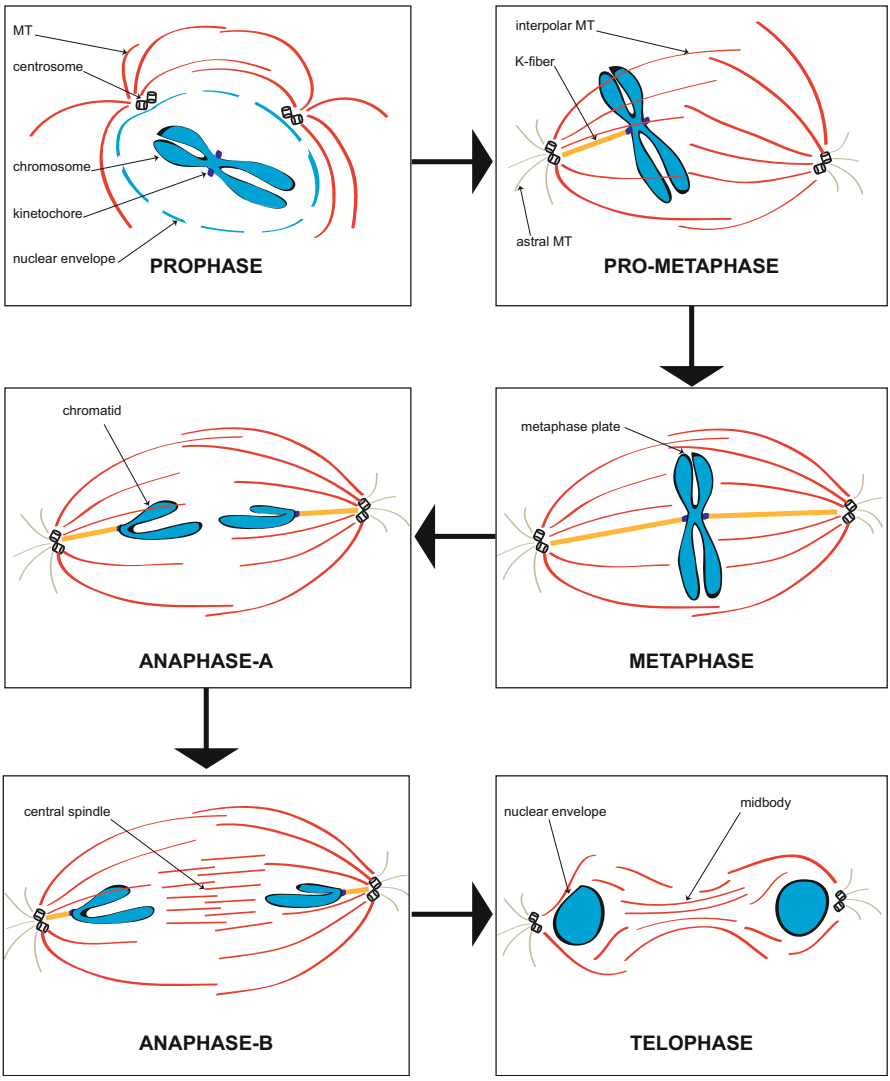

Fig. 1.2 Mitotic phases. In prophase, chromosomes are condensed, and centrosomes have been duplicated and nucleate highly dynamics MTs. Nuclear envelope breaks down. In prometaphase, MTs become specialized. Some of them interact with the cell cortex and are called astral MTs; interpolar MTs form the main part of the bipolar spindle and MTs interacting with the kinetochores become organized into K-fibers. In metaphase, the bipolar spindle is mature and the chromosomes are aligned in the center at the metaphase plate, all of them connected to the two opposite poles through sister K-fibers. In anaphase A, K-fibers shorten and pull the sister chromatids apart. In anaphase B, a new MT-based structure, called the central spindle, is built between the two chromosome masses and helps in separating them. In telophase, nuclear envelope forms again and the midbody will define the site of abscission between the two daughter cells

and modeling approaches have suggested that a process involving only centrosomal MTs could not account for the rapid attachment of all chromosomes in human cells in the observed time (Wollman et al. 2005). Indeed, we know now that in addition to the centrosomes, other pathways are set up in mitotic cells to promote MT nucleation and assembly in an acentrosomal manner. We will see later in this chapter that these mitotic pathways are essential for spindle assembly. We will also discuss whether their role is merely to provide an efficient "search and capture" mechanism or whether it may go beyond.

1.3 General Principles in Mitotic Spindle Assembly

Since the bipolar spindle is constituted by transient interactions between highly dynamic MTs, it is itself highly dynamic in nature, a property that underlies its self-organization and self-correction properties. In the most extreme case, mitotic cells can rebuild a spindle after the full depolymerization of MTs by cold or drug treatments (Tulu et al. 2003). Under physiological conditions, spindle dynamics enables the correction of erroneous MT-chromosome attachments thereby preventing defects in chromosome segregation or mitotic slippage. However, and despite its highly dynamic nature, the spindle also has to provide mechanical support for the forces required to move and segregate the chromosomes.

In this chapter, we will focus on two general mechanisms essential to understand how MTs organize the bipolar spindle: First, we will focus on the mechanism by which cells promote the assembly of a large number of highly dynamic MTs by upregulating MT nucleation. Second, we will focus on the global and local regulation of MT dynamics.

1.3.1 Control of MT Nucleation in M Phase

In cells, the tubulin concentration does not reach the critical threshold for spontaneous MT assembly. In fact, cells define and control where and when MT assembly occurs by using a specific mechanism driving MT nucleation. In eukaryotic cells, this involves a major MT nucleation complex called the γ-TuRC (γ-tubulin ring complex). This protein complex is composed by multiple copies of γ-tubulin and five additional proteins called GCPs (γ-tubulin complex proteins) that organize a ringlike structure postulated to act as a template for tubulin dimer addition and MT polymerization (Teixido-Travesa et al. 2010; Kollman et al. 2011). A number of additional proteins associate with the γ-TuRC. One of them, NEDD1 (also called GCP-WD), functions as an adaptor or targeting factor for the γ-TuRC (Haren et al. 2006; Luders et al. 2006; Zhu et al. 2008; Zhang et al. 2009; Sdelci et al. 2012; Pinyol et al. 2013). For a detailed description of this complex and its role in MT nucleation, we refer the reader to excellent reviews (Kollman et al. 2011; Teixido-Travesa et al. 2012) (also see the Chap. 4 by Sánchez-Huertas, Freixo, and Lüders).

In mitosis, MT nucleation increases through different mechanisms that all involve the γ-TuRC (Moudjou et al. 1996; Teixido-Travesa et al. 2012). This in turn defines different MT assembly pathways.

1.3.1.1 Centrosome Maturation and MT Nucleation

Centrosomes are MT-based organelles playing a variety of functions in the cell (Bettencourt-Dias and Glover 2007). In higher eukaryotes, the cell has one centrosome composed by a pair of centrioles surrounded by pericentriolar material (PCM) (see the Chap. 3 by Comartin and Pelletier for a detailed description). In interphase, the centrosome is the main MT organizing center (MTOC), promoting MT nucleation and maintaining in focus most MT minus ends. In cycling cells, the centrosome duplicates during interphase and before mitosis onset, the duplicated centrosomes undergo maturation characterized by the active recruitment of PCM components, in particular MT nucleation factors such as γ-tubulin as part of the γ-TuRC (Khodjakov and Rieder 1999; Piehl et al. 2004). Centrosome maturation therefore leads to a dramatic increase of the MT nucleation activity. This promotes the formation of two asters of dynamic MTs that get positioned on opposite sides of the nucleus through the active separation of the centrosomes before the nuclear envelope breaks down. However, centrosomes are not essential for bipolar spindle assembly (Bettencourt-Dias 2013). Other pathways are specifically set up in dividing cells and activate MT nucleation in a centrosome-independent manner.

1.3.1.2 Chromosome-Dependent MT Assembly

The last 20 years have provided compelling evidence for the existence of a specific MT nucleation and assembly pathway triggered around the chromosomes in dividing cells (Karsenti et al. 1984; Heald et al. 1996). The underlying mechanism involves the activity of Ran, a small GTPase that is essential for nucleocytoplasmic transport in interphase (Clarke and Zhang 2008). In dividing cells, the association of its guanosine exchange factor (GEF) RCC1 with the chromatin results in the formation of a GTP-bound Ran (RanGTP) gradient centered around the chromosomes (Carazo-Salas et al. 1999; Kalab et al. 1999, 2002; Ohba et al. 1999; Zhang et al. 1999; Carazo-Salas et al. 2001; Gruss et al. 2001). Following the same basic mechanism as in interphase, RanGTP induces the release of NLS (nuclear localization signal)-containing proteins from karyopherins. Some of these proteins perform essential functions in spindle assembly. A number of recent reviews cover the principles of the RanGTP gradient in mitosis as well as the current knowledge on the identity and function of RanGTP-regulated proteins (Karsenti and Vernos 2001; Meunier and Vernos 2012).

The mechanism by which RanGTP upregulates de novo MT nucleation in the vicinity of the chromosomes is however not fully understood yet. As for all the other pathways, it requires γ-TuRC activity (Groen et al. 2004; Luders et al. 2006), but it also requires the RanGTP-regulated protein TPX2 (targeting protein for Xklp2) (Wittmann et al. 2000; Gruss et al. 2001, 2002), a specific activator of the Aurora A kinase (Bayliss et al. 2003; Eyers et al. 2003; Tsai et al. 2003). Recently, it was shown that the specific phosphorylation of the γ-TuRC-associated protein

NEDD1 by the mitotic kinase Aurora A is essential for this pathway (Pinyol et al. 2013). TPX2 interacts with the mitotic kinase Aurora A and activates it in a RanGTP-dependent manner, promoting NEDD1 phosphorylation and thereby a mechanism for the activation of MT nucleation by RanGTP (Scrofani et al. 2015).

Although many data support a mechanism for RanGTP-/chromosomal-dependent MT nucleation without a predefined site, some reports on the localization of γ-TuRCs at the kinetochores suggest that MTs may also be nucleated at the kinetochore (Torosantucci et al. 2008; Mishra et al. 2010). However, it is still not clear whether such a direct kinetochore-dependent MT nucleation occurs or whether MTs are stabilized in this specific region of the chromosomes (Tulu et al. 2006; Maresca et al. 2009; Needleman et al. 2010) by the chromosomal passenger complex, located at the kinetochores (Sampath et al. 2004; Tseng et al. 2010). The putative nucleation of MTs at the kinetochore would however result in their "reversed" polarity with their minus end at the kinetochore and their plus end extending toward the spindle poles. This orientation has never been observed in animal cells (Euteneuer and McIntosh 1981; Euteneuer et al. 1983; Rieder 2005; Kitamura et al. 2010).

1.3.1.3 MT Amplification

In addition to the activation of MT nucleation at the centrosome and around the chromosomes, the recruitment of γ-TuRCs on pre-existing MTs drives an amplification mechanism that increases MT polymer amounts (Goshima et al. 2007, 2008; Lawo et al. 2009). This pathway relies on the augmin complex, constituted by eight proteins in humans (Lawo et al. 2009). The augmin complex binds the lattice of a pre-existing MT recruiting the γ-TuRC and promotes the nucleation and elongation of a new MT (Kamasaki et al. 2013). This results in MT branching and drives the efficient amplification of the whole MT mass during mitosis (Petry et al. 2013). Augmin-dependent MTs are then sorted toward the spindle poles (Lecland and Luders 2014). The augmin-dependent amplification pathway plays an important role during cell division. Indeed, the silencing of some of its components results in dramatic phenotypes (Uehara et al. 2009; Wainman et al. 2009; Petry et al. 2011). Recently, this pathway has been proposed to be intimately related with the chromosomal, RanGTP-dependent pathway of MT assembly (Petry et al. 2013).

Altogether, various mechanisms boost MT nucleation in the dividing cells actively promoting MT assembly. This activation does not occur simultaneously or at a single site; it starts at the centrosomes and then around the chromosomes and on pre-existing MTs. One common requirement is the γ-TuRC (Moudjou et al. 1996; Teixido-Travesa et al. 2012) and NEDD1 phosphorylation (Haren et al. 2006; Luders et al. 2006; Zhu et al. 2008; Zhang et al. 2009; Johmura et al. 2011; Gomez-Ferreria et al. 2012; Sdelci et al. 2012; Pinyol et al. 2013; Scrofani et al. 2015). This regulatory mechanism involves different mitotic kinases that target specific sites on NEDD1 but interestingly all in close proximity within the protein (Luders et al. 2006; Sdelci et al. 2012; Pinyol et al. 2013). However, the precise mechanisms by which these phosphorylation events participate in the activation of MT nucleation are still not understood.

1.3.2 Balancing High MT Dynamics

Spindle assembly requires abundant dynamic MTs, but their local stabilization is essential to generate a structure that is robust enough to provide support for chromosome movements yet flexible enough to correct erroneous interactions with the chromosomes to ensure their error-free segregation.

The global destabilization of the interphase MTs (turnover in the range of minutes to hours) is triggered by the cell cycle machinery through the activation of the Cdk1 kinase. However, the highly dynamic nature of the mitotic MTs (turnover in the range of seconds to a few minutes) is finely controlled by MT stabilizing and destabilizing activities. This general concept was validated in vitro. Indeed, mitotic MT dynamics could be mimicked in vitro by adding at a certain ratio two proteins with antagonizing MT stabilizing and destabilizing activities (Tournebize et al. 2000). These two proteins are XMAP215/chTOG and MCAK.

chTOG/XMAP215 is an MT-associated protein (MAP) conserved in all eukaryotes (Gard and Kirschner 1987; Vasquez et al. 1994); it binds very efficiently to MTs and promotes their assembly in vitro and in vivo (Brouhard et al. 2008). XMAP215 can bind along the whole MT lattice but has also been characterized as a plus-end binding protein, promoting MT polymerization. Indeed, MT stabilizing factors are MAPs acting through various mechanisms. Some of them as XMAP215 are found along the entire MT length, whereas others have specific plus-end-tracking properties (+TIPs). They localize to the MT growing ends, probably recognizing the GTP-bound state of tubulin, and regulate their dynamic behavior (Mimori-Kiyosue et al. 2000; Maiato et al. 2005; Kronja et al. 2009; Akhmanova and Steinmetz 2010; Maurer et al. 2012; Zanic et al. 2013; Alushin et al. 2014; Zhang et al. 2015). Finally, other MT stabilizing factors seem to function by protecting MT against depolymerization. At MT minus ends, the γ-TuRC or other minus-end binding complexes may stabilize MTs through an end-capping activity (Wiese and Zheng 2000; Goodwin and Vale 2010; Meunier and Vernos 2011; Jiang et al. 2014; Meunier et al. 2015).

Counteracting the action of MT stabilizing factors, three different types of activities promote MT destabilization: catastrophe factors, tubulin sequestering factors, and severing factors. MCAK is a major MT depolymerase belonging to the kinesin 13 family (constituted by KIF2A, KIF2B, and MCAK/KIF2C). In vitro, MCAK was shown to attach to the MT lattice through electrostatic interactions and diffuse along the MT, driving MT depolymerization both at MT plus and minus ends (Walczak et al. 1996; Desai et al. 1999; Hunter et al. 2003). The members of another class of depolymerizing kinesins in the kinesin 8 family (KIF18A and KIF18B) use a distinct mechanism to induce MT destabilization. They act by blocking the incorporation of new tubulin dimers at MT plus ends (Mayr et al. 2007; Du et al. 2010; Walczak et al. 2013). An additional way to regulate MT stability involves the protein Op18 (also called stathmin). Op18 binds free tubulin dimers and impairs their incorporation at the MT plus end (Belmont and Mitchison 1996; Cassimeris 2002; Gupta et al. 2013), leading to a decrease in MT growth and MT destabilization. Finally, a number of MT severing enzymes have

been recently identified, affecting MT stability within the mitotic spindle (Sharp and Ross 2012).

Altogether, a large number of mechanisms control MT stabilization and destabilization. They are tightly regulated and coordinated in space and time during mitosis to ensure the correct assembly and function of the bipolar spindle. A spatial control on MT dynamics is provided by the chromosomes through the RanGTP pathway that favors MT stabilization by creating a gradient of MT stabilizing factors (Karsenti and Vernos 2001; Caudron et al. 2005; Clarke and Zhang 2008). The RanGTP gradient is moreover translated into a phosphorylation gradient through the TPX2-dependent activation of the Aurora A kinase, promoting MT stabilization around the chromosomes (Eyers et al. 2003; Tsai et al. 2003; Sardon et al. 2008). The assembly of the full mitotic spindle appears therefore to rely on overlapping regulatory gradients. The RanGTP gradient around the chromosomes favors MT nucleation and stabilization, and the Aurora A kinase acts on MT stabilization at the chromosomes and the centrosomes. A Plk1-dependent phosphorylation gradient around the centrosomes favors MT assembly and is essential for spindle positioning (Kiyomitsu and Cheeseman 2012). An Aurora B-dependent phosphorylation gradient centered at the kinetochores in the first phases of mitosis and at the central spindle in anaphase is involved in multiple functions, including MT dynamic regulation (Carmena et al. 2012). Altogether, these regulatory gradients within the mitotic cell stabilize and orient the nascent MTs in order to organize MTs into a spindle-shaped structure, with MT minus ends focused at the two poles and their plus ends interdigitating or connecting with the chromosomes.

1.4 MT Organization in the Spindle: Different Configurations, Functions, and Properties

While keeping highly dynamic properties, MTs get organized into different structures whose properties and dynamics change throughout cell division. In this section, we will focus on the principles driving the organization of the three most characteristic mitotic MT assemblies: the bipolar spindle, the K-fibers, and the central spindle.

1.4.1 The Bipolar Spindle

The organization of MTs into two interdigitating antiparallel arrays is key to cell division. The main forces driving MT organization into this typical configuration are provided by molecular motors that interact with MTs in an ATP-dependent manner using the energy derived from its hydrolysis to move directionally along the MTs. While some of them move toward MT minus ends (dynein and some kinesins), others move toward the plus ends (most of the kinesins). Recent excellent reviews describe in detail the mechanochemistry of motor movement and force generation (Roberts et al. 2013; Cross and McAinsh 2014).

Mitosis in human cells involves the activity of a large number of kinesins and cytoplasmic dynein. The collective action of these motors drives bipolar spindle assembly by establishing and maintaining three main activities: stable but dynamic interactions between the two antiparallel MT asters, focusing MT minus ends into the spindle poles, and dynamic interactions between MTs and chromosomes. Each one of these functions is related to specific motor organizations and mechanisms of action. Motors can indeed cross-link and move on two antiparallel MTs, link two MTs but move on only one of them, or mediate the MT-chromosome interaction. We will here briefly describe examples of these three kinds of motors.

Eg5 (also called KIF11 or kinesin 5) is an extensively characterized mitotic motor. It is a homotetramer that can interact with two MTs preferentially in an antiparallel configuration. By moving toward the plus ends of the two cross-linked MTs, it drives their separation (van den Wildenberg et al. 2008; Tanenbaum and Medema 2010) (Fig. 1.3). This is an essential mechanism promoting spindle pole separation and bipolarity establishment and maintenance. Eg5 function is essential for bipolar spindle organization. In the absence of Eg5 activity, MTs organize into a monopolar spindle with the MT minus ends in the center and the plus ends at the periphery of a rosette-like structure (Mayer et al. 1999).

Other MT-cross-linking motors adopt distinct configurations, such as HSET, also known as KIFC1 or XCTK2 (Walczak et al. 1997; Mountain et al. 1999). HSET is a minus-end-directed kinesin, which cross-links two MTs, but moves only on one of them. It is organized as a homodimer that can interact with one MT through its motor domain and with another MT through another ATP-independent MT-binding domain, promoting MT organization (Fig. 1.3). HSET plays a role in spindle length control and, like the dynein complex, in spindle pole focusing (Cai et al. 2009; Hentrich and Surrey 2010). Dynein is a major minus-end-directed motor in the mitotic cell. Depending on its "cargo," dynein functions by promoting MT movement in relation to another MT (pole focusing), to the nuclear envelope (pole separation in prophase), to the cell cortex (spindle positioning), or to the kinetochore (chromosome positioning) (Roberts et al. 2013) (Fig. 1.3).

Another class of motors includes the chromokinesins that mediate interactions between MTs and the chromosome arms. The forces exerted by chromokinesins are called polar ejection forces that play important roles in chromosome congression (Vanneste et al. 2011). At least two classes of kinesins are able to directly interact with chromosome arms: KIF22 (Kid) and KIF4 (Mazumdar and Misteli 2005). The role of Kid in chromosome congression is essential (Levesque and Compton 2001). In Xenopus egg extracts, Xkid depletion results in the dramatic chromosome scattering phenotype (Antonio et al. 2000; Funabiki and Murray 2000). Chromokinesins share common structural features. They are all plus-end-directed motors and function in homodimers, with a motor MT-binding domain in N-terminal and a DNA-binding motif in the C-terminal part (Vanneste et al. 2011) (Fig. 1.3).

The complexity of motor functions and their interactions is overall a challenge to achieve a full understanding of their roles in bipolar spindle assembly. Their function cannot be limited to focusing minus ends at the poles, congressing

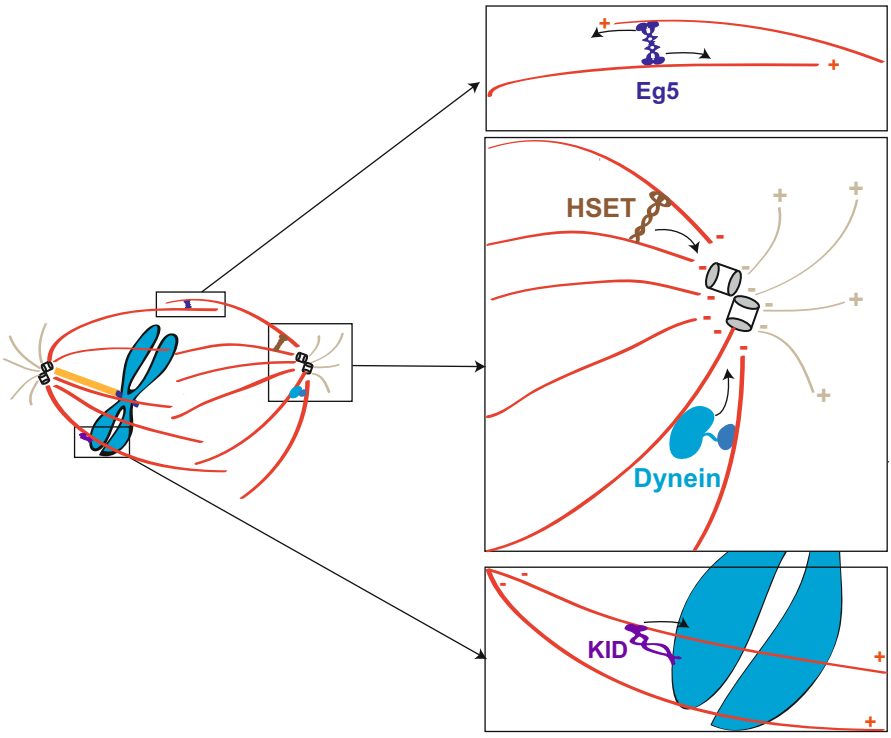

Fig. 1.3 Bipolar spindle MT organization. *Left*: schematic representation of a prometaphase spindle. *Right boxes*: (*upper box*) Eg5 is a homotetrameric, plus-end-directed motor. It works separating two antiparallel MTs apart and is essential for spindle bipolarization and cell division. *Middle box*: HSET and dynein are two types of minus-end-directed motors. They move on one MT and also interact with another, parallel, MT. This process is essential for focusing MT minus ends at the poles. *Lower box*: KID is one example of chromokinesins. It is a plus-end-directed, homodimeric motor interacting with an MT through its motor domain and with chromosomes through its C-terminal part. Its function is essential in congressing the chromosomes in the metaphase plate

chromosomes, and cross-linking antiparallel MTs in the region of their overlap. For example, dynein has also a role in the targeting of a number of essential factors to the spindle poles, including Eg5 and TPX2 (Ma et al. 2010).

1.4.2 The K-Fibers

As the spindle assembles, the dynamic plus ends of some MTs are "captured" by the kinetochore, a specialized region organized as a paired structure on each chromosome. MT plus-end attachment to the kinetochore is mediated by interactions with the Ndc80 and Ska complexes leading to their stabilization (Jeyaprakash et al. 2012; Cheerambathur et al. 2013; Shrestha and Draviam 2013). These MTs

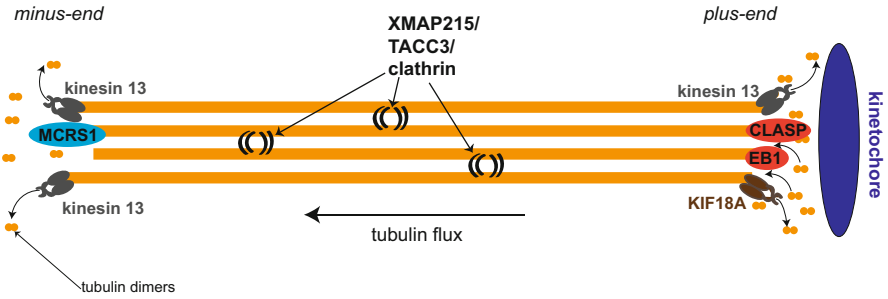

Fig. 1.4 K-fiber organization. K-fibers are bundles of 20–40 MTs. Bridges between MTs, in part composed by XMAP214, clathrin, and TACC3, maintain the MTs forming the K-fiber together. Their plus ends are interacting with the kinetochores, and their minus ends are focused in close proximity to the spindle poles. K-fibers are constantly depolymerizing at the minus end and overall incorporate new tubulin dimers at the plus end. This mechanism creates a tubulin poleward flux from the plus to the minus end of the K-fibers. While kinesins 13 work at both ends in depolymerizing K-fiber MTs, KIF18A is a depolymerizing kinesin specific for the plus end. At the plus end, +TIPS factors such as EB1 and CLASPs favor MT polymerization. At the minus end, MCRS1 protects MTs against destabilization

form bundles of 20–40 MTs organized in parallel orientation, called the K-fibers. K-fibers have specific dynamic properties and are remarkably more stable than the other spindle MTs (Rieder 1981). They generate pushing forces that contribute to centrosome separation and the establishment of spindle bipolarity and are obviously essential for chromosome movements and segregation (McHedlishvili et al. 2012).

The K-fiber MT bundles require protein complexes forming bridges in between the parallel MTs. One of them is composed by clathrin and TACC3 that interacts with the MT polymerase chTOG/XMAP215 (Booth et al. 2011) (Fig. 1.4). K-fiber organization in bundles is however still poorly understood, since other kinds of inter-MT bridges have been observed (Booth et al. 2011).

The K-fibers exhibit very specific plus- and minus-end dynamics that are tightly related to attachment error correction and chromosome movements. The MT plus ends of the K-fiber alternate between phases of growth and depolymerization that drive poleward and anti-poleward chromosome movements and oscillations that result in chromosome alignment at the metaphase plate (Magidson et al. 2011). While the plus ends exhibit a "switching" behavior alternating between phases of MT growth and shrinkage coordinated at the two sister kinetochores, K-fiber MT minus ends depolymerize constantly. This results in a characteristic tubulin flux from the plus end toward the minus end (Fig. 1.4) that generates forces within the spindle strong enough to move the chromosomes (Waters et al. 1996).

K-fiber dynamics regulation involves both proteins favoring MT polymerization like CLASP or EB1 and others promoting MT destabilization like the kinesin 13 and 8 family members (Tirnauer et al. 2002; Maiato et al. 2005; Joglekar et al. 2010; Manning et al. 2010) (Fig. 1.4). The mechanism involved in regulating MT depolymerization at the minus end is still unclear. However, the recent identification of novel proteins that specifically protect MT minus ends from depolymerase

activities opens the way to a better understanding of K-fiber dynamics (Goodwin and Vale 2010; Meunier and Vernos 2011; Jiang et al. 2014; Meunier et al. 2015) (Fig. 1.4).

A dramatic change in K-fiber dynamics drives K-fiber shortening and thereby chromosome segregation in anaphase (Waters et al. 1996). However, how the shortening of all K-fibers is coordinated, which signal triggers of the process, and what mechanism controls the depolymerization rate are still open questions.

1.4.3 The Central Spindle

During anaphase, a new MT-based structure, called the central spindle, assembles between the two segregating chromosome masses. The central spindle is formed by interdigitating MT arrays that promote the separation of the centrosomes and chromosomes by sliding in an antiparallel manner. MTs in the central spindle are organized into bundles that are remarkably more stable than those forming the metaphase spindle (Saxton and McIntosh 1987).

The mechanism underlying central spindle assembly involves at least two protein complexes. One of them called, centralspindlin, initiates central spindle assembly. It is formed by a kinesin-like protein, MKLP1, and a Rho GTPase-activating protein (RhoGAP), CYK-4. This protein complex is targeted to an antiparallel MT overlapping area immediately after chromosome segregation (Mishima et al. 2002; White and Glotzer 2012) (Fig. 1.5). Centralspindlin plays also essential roles in midbody assembly and abscission, the very last steps in the full separation of the daughter cells.

The mechanism defining the extent of MT overlap and sliding in the central spindle involves another complex constituted by the protein PRC1 (protein

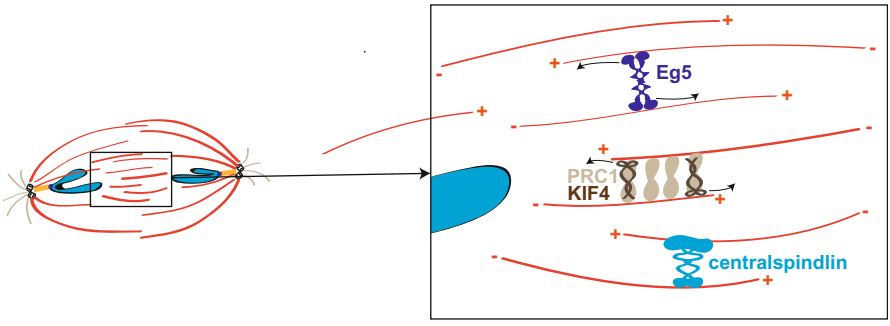

Fig. 1.5 Central spindle organization. *Left*: schematic representation of an anaphase B spindle, with the central spindle in the center. *Right box*: detail of central spindle organization. MTs plus tips are interdigitating in the central spindle. They are cross-linked together though the action of two main complexes: the centralspindlin complex and the complex formed by PRC1 and KIF4. The interaction between PRC1 and KIF4 is essential for maintaining central spindle length and defining the antiparallel, overlapping MT region

regulator of cytokinesis 1), a MAP with MT bundling activity in vitro (Mollinari et al. 2002), and the kinesin KIF4 (Kurasawa et al. 2004) (Fig. 1.5). In vitro experiments with recombinant PRC1 and KIF4 showed that these two activities are sufficient to reconstitute a central spindle-like organization (Bieling et al. 2010).

Several regulating protein complexes are targeted to the central spindle during anaphase. Among them, the chromosomal passenger complex (CPC) containing the Aurora B kinase relocalizes from the kinetochores to the central spindle and regulates central spindle assembly and function, by phosphorylating a variety of substrates (Guse et al. 2005). Recently, it was also shown that the kinase Aurora A plays a role in central spindle organization and dynamics regulation through the phosphorylation of the dynein complex and TACC3 (Lioutas and Vernos 2013; Reboutier et al. 2013).

The central spindle is constituted by some interpolar MTs, but recent studies have demonstrated that it can be assembled de novo, involving MT nucleation and stabilization. The signals triggering the assembly of these MTs are however still not identified, but the involvement of the RanGTP pathway and the augmin complex is likely (Glotzer 2009; Uehara and Goshima 2010).

1.5 Specifying MT Identities in the Spindle?

MTs during mitosis show a range of dynamic properties, organization, and function that coexist at any given time and evolve as mitosis proceeds. Overall, astral and interpolar MTs are very dynamic mainly at their plus ends. K-fibers are organized into bundles with specific dynamic properties both at the plus and at the minus ends, while being more stable than the other spindle MTs. How these characteristics are specified is still not understood. In fact, it is unclear whether MT organization determines function (for instance, MTs contacting the kinetochore organize into K-fibers) or whether different MTs with specific properties define organization and function. In this context, it may be relevant to consider that mitotic MTs originate at different sites and through different pathways that involve specific components and regulators. Interestingly, some proteins were found to specifically associate with one class of MTs. The RanGTP-regulated protein HURP associates only with the K-fibers in a region close to the chromosomes (Sillje et al. 2006). Another RanGTP-regulated protein, MCRS1, associates exclusively to chromosomal MTs and to those forming the K-fibers (Meunier and Vernos 2011). These data suggest that the chromosomal MTs participate at least in part to K-fiber formation, as they have specific MAPs that confer them properties different to the other MTs. This hypothesis still requires experimental support to be confirmed.

Other mechanisms could potentially confer specific properties to the spindle MTs. Although all MTs are formed by α-/β-tubulin dimers, mammalian cells have several genes for these two proteins (7 α-tubulin and 8 β-tubulin genes in humans). The different tubulin isotypes are extremely conserved (more than 95 % amino acid sequence identity), but their C-terminal tails have more variability. Interestingly, this region is exposed on the MT surface and is responsible for binding MAPs and

motors (Sirajuddin et al. 2014). The expression of specific tubulin isotypes has been related with specific MT organizations (Raff et al. 1997) and with adaptation mechanisms. Although it is not yet clear whether they play any role in mitosis, changes in the expression pattern of tubulin isotypes have been reported in some cancer cells that show altered MT dynamics and resistance to antitumor treatments that target tubulin (Wang et al. 2014).

In addition to the expression of specific tubulin isotypes, several posttranslational modifications (PTMs) on the C-terminal tails of the tubulins modulate the binding affinities of MAPs and motors and may even change motor processivity and/or velocity (Janke and Bulinski 2011; Sirajuddin et al. 2014). Many PTMs have been described including detyrosination, mono- or poly-glutamylation, phosphorylation, acetylation, and glycylation. These modifications may constitute a "tubulin code" that could specify which MAPs and which motors would bind to one specific MT (Janke and Bulinski 2011; Magiera and Janke 2014; Barisic et al. 2015). Although this remains speculative, the number of α- and β-tubulin isotypes together with the combinatorial possibilities of PTMs at their C-terminus potentially offers a myriad of possibilities to precisely define the properties of MT subpopulations, and this could play a role in the bipolar spindle.

1.6 Mitotic MTs in Health and Disease

1.6.1 Mitotic MT-Related Disorders and Pathologies

Cell division is fundamental for life. Any error in this process may be fatal or generate cells with an incorrect chromosome number. Aneuploidy, the loss or gain of chromosomes, is the leading genetic cause of miscarriage and congenital birth defects as well as being tightly associated to health-threatening conditions like cancer.

In humans, as many as one in five pregnancies end in miscarriage, the most common complication of early pregnancy. Aneuploidy is the leading known cause of miscarriage, but some of them (as, e.g. trisomy 21 and monosomy X, Down or Turner syndromes, respectively) are compatible with live birth, making aneuploidy the leading cause of congenital birth defects and mental retardation. A common cause for these miscarriages seems to be aneuploidies in human oocytes, which increase dramatically with age (Holubcova et al. 2015). This is associated to the weakening of cohesion between the sister chromatids with time (Chiang et al. 2010; Duncan et al. 2012). This phenomenon was recently described as "chromosome fatigue" (Daum et al. 2011) and is particularly threatening for human reproduction because oocytes are maintained blocked in prophase of meiosis I from birth until maturation is induced after puberty on a monthly basis throughout the reproductive lifespan (Chiang et al. 2010).

A number of pathologies derive from spindle orientation defects that compromise the fate of the daughter cells during development (Noatynska et al. 2012). During development, neural progenitor cells undergo symmetric and asymmetric

divisions from a monolayer of stem cells to build the brain. Mutations in genes related with centrosome function and duplication or with astral MT stabilization have been related to brain development defects like microcephaly or lissencephaly, respectively (Fish et al. 2006; Yingling et al. 2008; Chavali et al. 2014). However, the mutated genes have a large range of distinct functions in various processes and organs (Noatynska et al. 2012) and a direct causal relationship between spindle orientation defects and these brain pathologies is currently missing.

Cell division is essential after birth for the growth of organs and body parts and throughout adulthood for the maintenance and renewal of cells and tissues. Mutations in proteins related with mitotic MT regulation, for example, in the PCM component pericentrin, have been linked to a number of pathologies including cancer (Delaval and Doxsey 2010). Most human solid tumors have aneuploid cells due to CIN (chromosome instability), which promotes chromosome missegregation in mitosis. CIN occurs early in tumorigenesis and is associated with poor prognosis. Aneuploid cells may get supernumerary copies of oncogenes and/or insufficient copies of tumor suppressor genes, which could favor the development of tumors (Duijf and Benezra 2013; Salmela and Kallio 2013). CIN can be caused by multiple mechanisms including a weakened or overactivated mitotic spindle assembly checkpoint, sister chromatid cohesion defects, increased merotelic kinetochore-microtubule attachments, or the presence of extra centrosomes. Although CIN was proposed as a leading cause of tumor progression, recent studies suggest that CIN can either promote or suppress tumor progression, depending on the context.

Aneuploidy or other mechanisms may also be involved with changes in the expression levels of mitotic factors: enzymes involved in the regulation of the cell division like the kinase Aurora A as well as MT-binding proteins like TPX2. These two proteins interact during mitosis and the TPX2-Aurora A complex has been described as an "oncogenic holoenzyme" (Asteriti et al. 2010). Interestingly, TPX2 was found to be the protein with the highest CIN (chromosome instability) score among 10,000 analyzed genes in a number of tumors (Carter et al. 2006).

1.6.2 MTs and Therapeutic Strategies

The highly dynamic properties of the mitotic MTs are essential for the assembly of a functional spindle. At the same time, they render mitotic cells particularly sensitive to factors that alter these properties. In fact, MT-binding agents (TBAs) that alter MT dynamics were the first antitumor compounds used for cancer treatment. There are different classes of TBAs isolated from a broad range of species, such as bacteria, sponges, or plants. They either promote MT stabilization (such as taxanes and epothilones) or MT destabilization (such as vinblastine). The prevailing idea is that TBAs exert an anticancer activity by targeting dividing cells particularly abundant in tumors, although this is under debate (Mitchison 2012; Topham and Taylor 2013). In any case, these drugs are still widely used in the clinic as they have a clear therapeutic value. However, there is a need for novel ways to fight tumors, as cancer cells can evade the effects of compounds and drugs through

different mechanisms. Interestingly, one of them is the expression of the neuronal βIII-tubulin isotype (Cortes and Vidal 2011). Moreover, TBAs are not specific for the dividing cells, generating secondary effects like neurotoxicity (Harrison et al. 2009; Kavallaris 2010).

Targeting characteristics specific to tumor cells, such as CIN, aneuploidies, and supernumerary centrosomes, is therefore an attractive therapeutic avenue. Compounds targeting mitotic factors such as the kinases Plk1, Aurora A or Aurora B (Salmela and Kallio 2013), or microtubule motors have been developed. Some of them are in different phases of clinical trials (Ding et al. 2014).

1.7 Conclusions

Spindle assembly relies on the coordination of a number of individual events including MT nucleation, stabilization, and organization. The importance of accurate chromosome segregation for the continuity of life is underscored by tightly controlled mechanisms ensuring the interaction between MTs and chromosomes with several layers of regulations and checkpoints that ensure the fidelity of the system. However, errors in cell division can occur, often leading to catastrophic consequences in terms of fertility, development, or tissue maintenance and renewal. Although individual pieces of the puzzle start to be well understood, future work will certainly focus on getting more information on the coordination of all single events and thereby a global view of a system at the basis of life transmission.

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Non-centrosomal Microtubule Organization in Differentiated Cells

2

Vyacheslav Dyachuk, Christiane Bierkamp, and Andreas Merdes

Abstract

The centrosome consists of a pair of centrioles surrounded by pericentriolar material. During the formation of the mitotic spindle, multi-protein complexes in the pericentriolar material are involved in the nucleation and anchorage of microtubules. In postmitotic cells of many tissues, proteins of the pericentriolar material lose their association with the centrosome and redistribute to various sites in the cytoplasm, to the cellular cortex, or to the nuclear surface. Consequently, the organization of the microtubule network is changed. Localization of centrosomal proteins and organization of microtubules follow cell type-specific patterns, to fulfill specialized functions. For example, in polarized epithelia, microtubules are involved in transcytosis and establishment of epithelial polarity, in neurons microtubules are necessary for axonal transport, or in muscle microtubules participate in the assembly of sarcomeres and in the positioning of nuclei. In this review, the principles of microtubule organization in different cell types will be described. The role of microtubules in muscle cells and the potential involvement of microtubule-dependent processes in muscular diseases will be documented in detail.

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2.1 Introduction

During division of somatic animal cells, centrosomes serve as centers for the nucleation and organization of microtubules. At each pole of the mitotic spindle, a pair of centrioles can be found, surrounded by pericentriolar material from which microtubules emanate. After completion of mitosis, the involvement of the centrosome in microtubule organization varies among different cell types. Although textbook illustrations often depict the centrosome as an organizing center of a radial microtubule network in interphase, this type of microtubule organization may only reflect a special situation seen in two-dimensional cell culture. In animals, radial organization of microtubules from the centrosome may still be detected in fibroblasts or in other migratory cell types, but in differentiated cells of many tissues, the centrosome loses its role as an organizing center. The following chapter will highlight several typical examples of non-centrosomal microtubule organization, with a particular focus on the transformation of the microtubule network in differentiating muscle cells. The potential role of microtubules in muscular tissue and potential defects in myopathies will be discussed.

2.2 Microtubule Organization in Polarized Epithelia

Among the numerous cell types that exhibit altered microtubule organization after differentiation, perhaps the best-studied objects are polarized epithelial cells of various origins. Monolayers of polarized epithelial cells are connected by intercellular connections, such as tight junctions, that form an impermeable barrier. These tight junctions separate the plasma membrane into an apical domain and a basolateral domain. Any transport processes across the epithelial layer have to occur via transcytosis, involving directed intracellular transport. Consequently, microtubules in these cells are organized in a polarized manner and serve as tracks for polarized transport. Pioneering studies on *Drosophila* wing epidermal cells revealed a uniform polarity of the microtubule cytoskeleton, with microtubule minus ends anchored in a region underlying the apical plasma membrane and plus ends terminating in the basal region of the cell (Mogensen et al. 1989). Similar organizational principles have been described for the microtubule network in other polarized epithelia, including cells from canine kidney, human intestine, rodent cochlea, *Drosophila* ommatidia, or *Drosophila* tracheal placodes (Bacallao et al. 1989; Meads and Schroer 1995; Tucker et al. 1992; Mogensen et al. 1993; Brodu et al. 2010). In all these cell types, centrioles are still visible in the apical area of the cytoplasm, but they no longer serve as major anchorage points for microtubules. Likewise, marker proteins of the centrosome are partly delocalized from the pericentriolar material. Whereas large amounts of gamma-tubulin and pericentrin are still focused around the centrioles of several epithelial cell types (Meads and Schroer 1995; Tucker et al. 1998), an increasing percentage of ninein in cochlear epithelial cells is lost from the centrosome during differentiation and accumulates at the non-centrosomal sites in the apical region of the cell. This is

particularly well visible in inner pillar cells of the organ of Corti in the cochlea, where ninein concentrates at the apical cell periphery in a ring-shaped area underlying the plasma membrane, where thousands of microtubule minus ends terminate (Mogensen et al. 2000). Since ninein is considered to play a role in anchoring microtubule minus ends (Dammermann and Merdes 2002), the data in cochlear epithelial cells have evoked the hypothesis of initial microtubule nucleation at the pericentriolar material, followed by release, translocation, and subsequent capture of microtubule minus ends at non-centrosomal apical sites containing ninein (Mogensen 1999). Release of microtubules from the centrosome in epithelial cells may involve microtubule-severing enzymes, such as spastin (Brodu et al. 2010). The maintenance of the non-centrosomal microtubule network may be further supported by a recently identified class of minus end-binding proteins termed CAMSAP, nezha, or patronin (Tanaka et al. 2012). Interestingly, a recent report on a ninein-related protein in the nematode *Caenorhabditis elegans*, NOCA-1, has shown functional redundancy with patronin, in the organization of non-centrosomal microtubule arrays in larval epidermal cells (Wang et al. 2015).

Along the basal cortex of polarized epithelial cells of the MDCK line, a separate set of microtubules has been described in addition to the apicobasal fibers, consisting of acentrosomal microtubules of mixed polarity that intersect and that interact with the cortex (Reilein et al. 2005). Small amounts of the microtubule-nucleating protein gamma-tubulin are found at branch points within this basal microtubule network.

In WIF-B cells that possess characteristics of polarized hepatocytes, neighboring cells are in close contact, except for small intercellular spaces that represent bile canaliculi (Ihrke et al. 1993). The plasma membrane surfaces outlining the bile canaliculi are equivalent to the apical membrane domains seen in columnar epithelial cells, such as intestinal or renal epithelia. In an analogous manner, gamma-tubulin is enriched underneath these membrane areas, from which microtubules radiate out toward the basolateral regions of the cells (Ihrke et al. 1993) (Fig. 2.1).

2.3 Microtubule Organization in Skin Keratinocytes

The epidermis of vertebrate skin is a stratified epithelium, i.e., an epithelium containing multiple layers of cells. The innermost layer is the "basal layer," in contact with the basement membrane, and consists of cells that maintain proliferative activity. Differentiating keratinocytes are oriented outward and establish a dense pattern of intercellular junctions, containing desmosomes, tight junctions, and adherens junctions. During differentiation, centrosomal proteins such as ninein lose their association with the pericentriolar material and redistribute to the cell cortex (Lechler and Fuchs 2007). Ninein interacts with the desmosomal protein desmoplakin. Moreover, adherens junctions have been found to be involved in microtubule reorganization: the protein p120/catenin has been shown to interact with microtubule plus ends in basal cells, via the plus end-binding protein CLASP2 (Shahbazi et al. 2013). Other microtubule-binding proteins, such as Lis1, Ndel1,

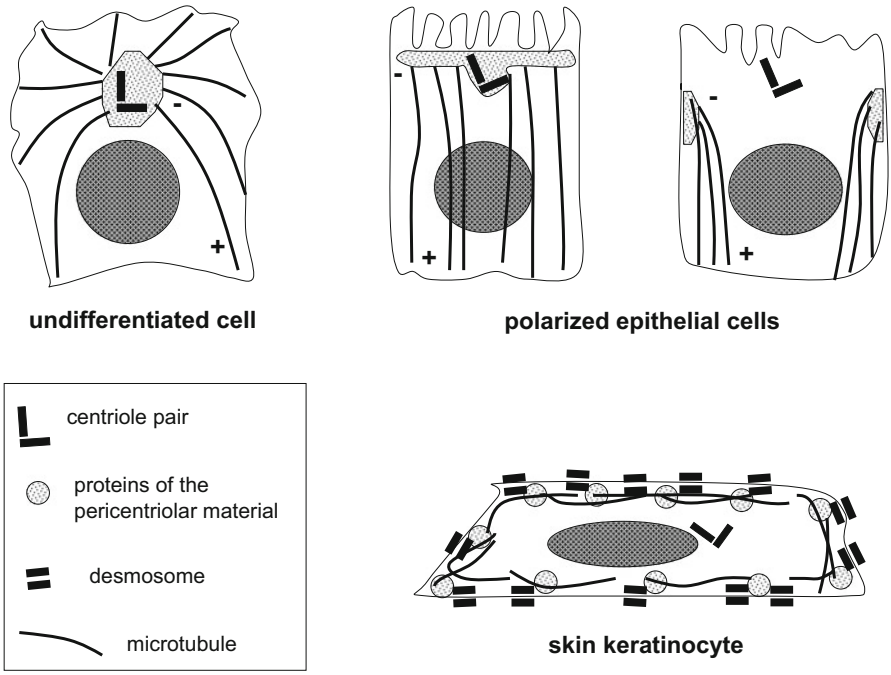

Fig. 2.1 Microtubule organization in epithelial cells. *Top left*: undifferentiated cell, with microtubules organized in a radial network. Microtubule minus ends are anchored at the pericentriolar material; plus ends are growing outward to the cell periphery. *Top middle and right*: two different examples for microtubule organization in polarized epithelial cells. *Top middle*: proteins of the pericentriolar material are redistributed to a wider apical region where microtubule minus ends are anchored. This resembles microtubule organization as seen in MDCK cells. *Top right*: Microtubule organization from specific sites underlying the apical plasma membrane, such as seen in cochlear epithelial cells. *Bottom*: microtubule organization in differentiated skin keratinocytes. Centrosome proteins such as ninein are relocalized to the cortex of the cell, binding to proteins of the desmosome. Microtubules concentrate in the cortical region of the cell

and CLIP170, equally associate with the cortex (Sumigray et al. 2011). The microtubule network is transformed during this process, from a centrosomally anchored network into a cortical array of fibers (Lechler and Fuchs 2007; Sumigray et al. 2011, 2012). Consistently, in differentiated keratinocytes of the suprabasal layer, the non-centrosomal minus end-binding protein Nezha equally localizes to the cortex (Shahbazi et al. 2013). Apparently, the density of desmosomal cell junctions and the cortical recruitment of microtubule-binding proteins are mutually dependent, as knockout of desmoplakin prevents cortical accumulation of ninein, Lis1, and Ndel1, and likewise the knockout of Lis1 provokes desmosomal defects with reduced desmosomal stability (Sumigray et al. 2011). A similar interdependence as seen for microtubules and desmosomes has been described for adherens junctions and microtubule plus ends (Shahbazi et al. 2013). Moreover, it has been

shown that the rapid incorporation of the desmosomal components Dsc2 and Dgl2 into desmosomes involves microtubule-dependent transport by the motor proteins KIF3A and kinesin 1, respectively (Nekrasova et al. 2011).

In primary cultures of keratinocytes, the formation of cell junctions and the transformation of the microtubule network can be followed upon induction of "differentiation" in vitro, by adding calcium to the culture medium. In such cultures, centrosomes maintain their ability to nucleate microtubules, but after initial nucleation, microtubules don't remain anchored at the pericentriolar material and redistribute in the cell (Lechler and Fuchs 2007). This observation is consistent with the "release and capture" model of microtubules that has been described for polarized epithelial cells (Mogensen 1999). Ninein could play the role of a crucial factor for anchoring microtubule minus ends to the centrosome in non-differentiated cells and to specific non-centrosomal sites after differentiation, although it still remains to be determined experimentally whether ninein takes indeed an active role in microtubule anchoring or whether it simply follows the reorganized microtubule network.

2.4 Microtubule Organization in Neurons

Neurons have a very specific morphology, with a main cell body (soma) from which one axon and multiple dendrites emanate. Within the soma, a pair of centrioles is located next to the nucleus (Sharp et al. 1982), surrounded by centrosomal proteins such as gamma-tubulin, pericentrin, or ninein (Baas and Joshi 1992; Leask et al. 1997; Baird et al. 2004). Despite the presence of a centrosome, microtubule ends are not anchored at the pericentriolar material but are found free in the cytoplasm (Baas and Joshi 1992). Arrays of overlapping microtubules are found along the length of axons and dendrites. These microtubules don't seem to have any specific points of anchorage, nor do their ends seem to be capped by gamma-tubulin complexes (Baas and Joshi 1992). Axons possess microtubules of uniform polarity, with the minus ends oriented toward the soma and the plus ends oriented toward the growth cone of the axon (Heidemann et al. 1981). Microtubules in dendrites, on the other hand, are of mixed polarity (Baas et al. 1988; Burton 1988). It is hypothesized that during neurogenesis, microtubules are initially nucleated at the centrosome, followed by release and translocation into the extending axon and dendrites. The release of centrosomal microtubules may involve microtubule-severing proteins, such as katanin and spastin (Ahmad et al. 1999; Wood et al. 2006). The position of the centrosome within the cell body is important for neuronal polarization, by defining the site of initial axon formation (de Anda et al. 2005). Experimentally generated neurons with multiple centrosomes grew additional axons in the vicinity of each centrosome, as verified by immunolabeling of the axon-specific microtubule-associated protein tau (de Anda et al. 2005). Interestingly, at later stages of neuronal differentiation, during the formation of synaptic connections, the centrosome may no longer function as a nucleation site, since gamma-tubulin has been found to be absent in synaptically coupled neurons in the hypothalamus and cortex

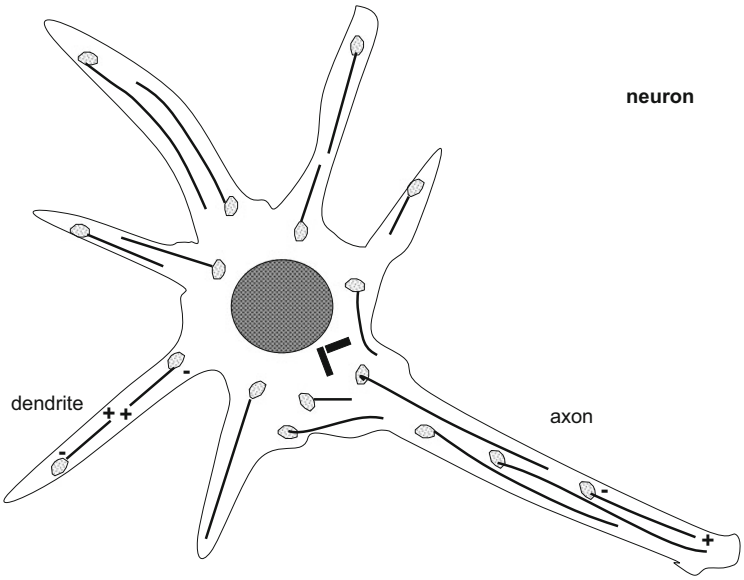

Fig. 2.2 Microtubule organization in neurons. Most microtubules are detached from the centrosome. Microtubules in the axon have a uniform polarity, with minus ends oriented toward the cell body and distal plus ends oriented toward the axonal growth cone. In dendrites, microtubules are of mixed polarity, with a subset of microtubules growing from distal sites toward the cell body. Proteins such as ninein are found widespread in the cytoplasm

(Leask et al. 1997). Moreover, in *Drosophila* neurons, the nucleation and organization of microtubules may not involve the centrosome at all (Nguyen et al. 2011). It is likely that a large number of microtubules are nucleated from cytoplasmic sites, or from the surface of existing microtubules, in particular at later stages. Specifically those microtubules in dendrites that grow from distal sites toward the cell body must originate from non-centrosomal sites, since their polarity is opposite to the polarity of microtubules growing outward from sites at or near the centrosome. Cytoplasmic microtubules may be anchored or stabilized by non-centrosomal ninein that has been detected in small particles, widespread in neurons (Baird et al. 2004). Additional information on microtubule organization in neurons can be found in the Chap. 4 by Sánchez-Huertas, Freixo, and Lüders (Fig. 2.2).

2.5 Microtubule Organization in Skeletal Muscle Cells

The microtubule network in skeletal muscle cells has been studied largely in cultures of myoblasts that undergo differentiation into myotubes upon serum starvation. Undifferentiated myoblasts possess a regular centrosome that acts as a nucleation center and that constitutes an anchorage point of a radial microtubule network. During differentiation, myoblasts elongate and subsequently fuse into

multinucleated, syncytial myotubes. In an early phase of the differentiation process, prior to fusion, proteins of the pericentriolar material accumulate at the cytoplasmic surface of the nuclear envelope (Tassin et al. 1985; Musa et al. 2003; Bugnard et al. 2005; Srsen et al. 2009). How pericentriolar proteins anchor to the nucleus is currently unknown. During myogenesis in *Drosophila*, RacGap50C, a protein that has been identified previously at the cleavage furrow of dividing cells, appears to be necessary for binding gamma-tubulin to various foci associated with the nuclear periphery (Guerin and Kramer 2009). Consistently, experiments involving regrowth of microtubules after previous depolymerization have shown that the nuclear surface can act as a nucleation center (Tassin et al. 1985; Bugnard et al. 2005; Fant et al. 2009). The centrioles are still detectable in fused myotubes during the first days of culture (Tassin et al. 1985), but individual marker proteins may be lost from the pericentriolar material in prolonged cultures (Connolly et al. 1986). Although centrioles may be partially degraded in maturing muscle, few centriolar cylinders seem to persist in adult muscle tissue, as electron microscopy of diaphragm muscle from rodents has revealed the presence of occasional centriole pairs (Kano et al. 1991). Differentiated myotubes in culture contain long, parallel arrays of microtubules, oriented along the long axis of the syncytial cell (Tassin et al. 1985). The formation of elongated microtubules depends on proteins of the EB family that are necessary for the shape of the cells and for fusion of myoblasts into myotubes (Straube and Merdes 2007; Zhang et al. 2009a). Although centrosomal proteins have largely accumulated at the nuclear surface in myotubes, most longitudinally oriented microtubules do not seem to be anchored to the nuclei in these cells (Musa et al. 2003). It is possible that the nuclear surface is involved in microtubule nucleation at an early stage of differentiation (Fant et al. 2009) and that microtubules are subsequently released and re-oriented in the cytoplasm, in a similar manner as seen in other differentiated cell types (see previous paragraphs). In muscle fibers from adult mouse tissue, a grid-like network of microtubules has been described (Kano et al. 1991; Ralston et al. 1999, 2001; Oddoux et al. 2013).

In these mature muscle fibers, the cytoplasm is filled with actin and myosin filaments that are organized into sarcomeres. Nuclei and microtubules are distributed in a thin cytoplasmic layer at the periphery of the fiber, and grids of orthogonally oriented microtubules are nucleated from elements of the Golgi complex. Clusters of the centrosome proteins gamma-tubulin and pericentrin co-localize with these Golgi elements (Oddoux et al. 2013). Growing microtubules are often guided by existing microtubules, with which they form bundles. At least in part, microtubules are also guided by dystrophin (Percival et al. 2007; Prins et al. 2009; Oddoux et al. 2013).

Interestingly, slight differences in microtubule organization exist between slow-twitch and fast-twitch fibers: in the former, bundles of microtubules are seen between nuclei, with few clear nucleation points. In the latter, more individual microtubules than bundles are visible, and these microtubules possess astral nucleation points adjacent to the nuclei (Ralston et al. 1999). The microtubule patterns in both slow- and fast-twitch muscle fibers can be slightly altered by experimental

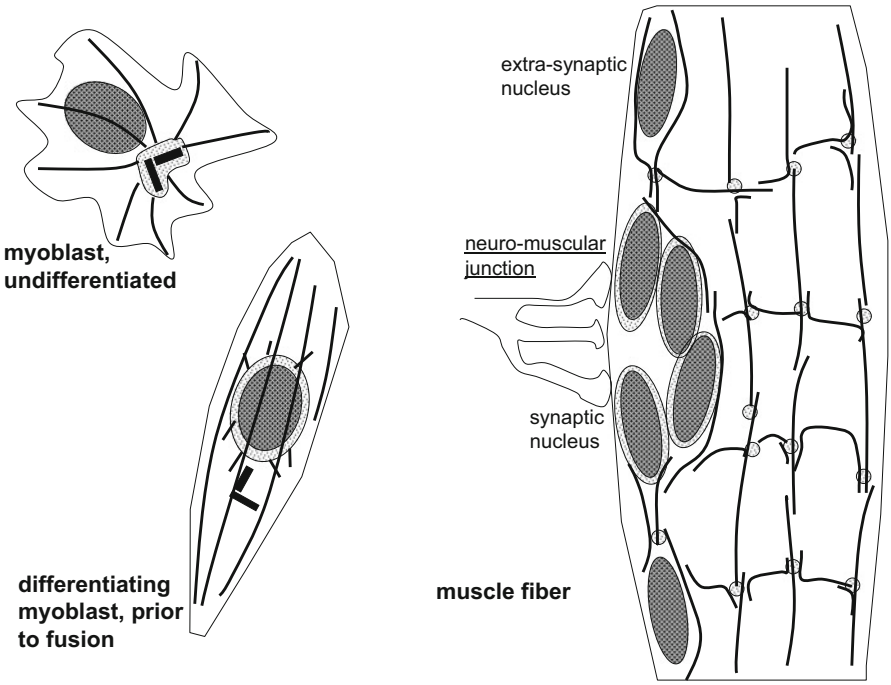

Fig. 2.3 Microtubule organization in muscle cells. In undifferentiated myoblasts, a fully functional centrosome is visible that acts as a microtubule-organizing center. At the beginning of the differentiation process, myoblasts elongate, and proteins of the pericentriolar material accumulate on the surface of the nucleus. At this stage, most microtubules are visible in long parallel arrays in the cytoplasm. Upon fusion of myoblasts into myotubes, actin and myosin organize into sarcomeres (not shown). In the fully mature muscle fibers, the microtubules are organized into a grid-like pattern, excluded from the sarcomeres. Elements of the Golgi complex associate with centrosome proteins and act as microtubule-organizing centers. The nuclei are pushed toward the periphery of the muscle fiber. Multiple synaptic nuclei are clustered near the neuromuscular junction, whereas the remaining extra-synaptic nuclei are distributed along the length of the fiber

stimulation with electrical pulses, mimicking different firing patterns of motor neurons (Ralston et al. 2001) (Fig. 2.3).

2.6 Potential Role of the Microtubule Network in Skeletal Muscle Cells

Skeletal muscle fibers are highly specialized cells that fulfill the single role of contraction and relaxation. They are postmitotic and thus unable to renew after injury. Damaged fibers are replaced by satellite cells that differentiate into new muscle cells. Since muscle fibers don't undergo any cell division, the question arises as to what specific role microtubules might play in differentiation and in the mature cell.

In early pharmacological experiments on cultures of myoblasts and myotubes, it has been shown that microtubules influence the distribution of intermediate filaments and myosin (Holtzer et al. 1975; Antin et al. 1981; Saitoh et al. 1988). More recently, direct evidence was obtained from microscopic observations of living myotubes that microtubules provide a scaffold for the transport of myosin and for the proper assembly of sarcomeres (Pizon et al. 2005).

Microtubules seem to play a second major role in myotubes, in the positioning of nuclei (Folker and Baylies 2013). After fusion of mononucleated myoblasts into multinucleated myotubes, most nuclei are distributed at equidistance along the periphery of the myotube. However, a subgroup of nuclei is found clustered beneath the neuromuscular junction. Although the specific role of these "synaptic" nuclei is still unclear, it has been suggested that they are involved in the maintenance of the synapse, for example, by an increased transcriptional activity to express acetylcholine receptors and other constituents of the postsynaptic membrane (Klarsfeld et al. 1991; Sanes et al. 1991). For the correct positioning of synaptic and extrasynaptic nuclei, microtubules likely serve as tracks, for motor-dependent transport of the nuclei. The dependence of nuclear positioning on microtubules has been shown first in cultured myotubes, in which clusters of acetylcholine receptor were experimentally induced on the plasma membrane, by treating the culture with extracts of electrical tissue from *Torpedo* fish (Englander and Rubin 1987). Once clusters of acetylcholine receptor had formed, the nearest nuclei moved to the cluster and got immobilized there. The movement of these nuclei occurred in a microtubule-dependent manner, since the microtubule poison colchicine inhibited any nuclear movement, unlike the actin poison cytochalasin D that had no such effect. More recent experiments in vivo, in mice and in *Drosophila*, confirm a role of microtubules in the positioning of myonuclei (Bruusgaard et al. 2006; Elhanany-Tamir et al. 2012).

The nuclear movement is driven both by plus end- and minus end-directed microtubule motors and might involve additional microtubule-associated proteins. In *Drosophila*, nuclear movement has been shown to involve kinesin 1 "KIF5B," dynein, and MAP7 (Metzger et al. 2012; Folker et al. 2012, 2014). On the surface of moving nuclei, kinesin acts at the leading edge, whereas dynein acts at the lagging edge, in addition to cortically anchored dynein that generates pulling forces on microtubules (Folker et al. 2014). In mouse C2C12 cells, dynein, dynactin, as well as KIF5B have been detected on the nuclear envelope of myotube nuclei (Cadot et al. 2012; Wilson and Holzbaur 2012, 2015). Dynein and kinesin motor complexes are involved in linear translocation of nuclei along microtubules and also in the rotation of these nuclei (Wilson and Holzbaur 2012).

Altogether, these experiments suggest that microtubules play an important role in the differentiation of myotubes, during the formation of sarcomeres and during the positioning of nuclei. This raises the question whether cellular defects in any known myopathies correlate with defects in the microtubule network.

2.7 Possible Involvement of Microtubules in Muscular Defects

Duchenne muscular dystrophy is one of the most abundant muscular diseases in children. Since the locus for this recessive muscular dystrophy is on the X chromosome, encoding the protein dystrophin, mainly boys are affected. Dystrophin is a large cytoplasmic protein that links the cytoskeleton to a complex of plasma membrane proteins, including alpha- and beta-dystroglycan that are connected to the extracellular matrix. Besides binding to the actin cytoskeleton, dystrophin has also been shown to interact with microtubules (Prins et al. 2009). Interestingly, the dystrophin-deficient *mdx* mouse that is considered an animal model for Duchenne muscular dystrophy shows disorganization of microtubules in skeletal muscle (Percival et al. 2007). The grid pattern of orthogonally oriented microtubules is lost in these mutant mice. At the same time, elements of the Golgi complex are distributed abnormally. The organization of microtubules as well as the distribution of Golgi elements can be largely restored by the expression of microdystrophin, a designed form of dystrophin lacking most of the central rod domain and the carboxy-terminus (Percival et al. 2007).

Besides showing differences in microtubule organization, muscle fibers of Duchenne muscular dystrophy appear to exhibit another defect involving microtubules: biopsies from patients display an increased percentage of myofibers with incorrectly positioned nuclei. Instead of localizing along the periphery, nuclei concentrate centrally within the fiber (Bell and Conen 1968). This unusual pattern has been described in a variety of muscular diseases, also including Becker muscular dystrophy and Emery-Dreifuss muscular dystrophy (Folker and Baylies 2013). However, the significance of nuclear positioning for pathogenesis remains unclear.

In recent years, the molecular mechanisms leading to defective distribution of nuclei have been tested in mutant mice with phenotypes resembling Emery-Dreifuss muscular dystrophy (Puckelwartz et al. 2009; Zhang et al. 2007a, 2010). In these mice, exons of the syne-1 gene were removed, encoding various parts of the carboxy-terminal region of the protein nesprin 1. As a consequence, these mice showed defects in the positioning of synaptic and extra-synaptic nuclei in skeletal muscle. Nesprins are a family of nuclear envelope proteins that provide a link between the cytoplasm and the inner nuclear membrane. Four nesprin genes exist in mammals, encoding proteins with a conserved carboxy-terminal "KASH" domain that binds to "SUN" proteins in the perinuclear space, i.e., in the lumen between the outer and inner nuclear membrane. The SUN proteins are transmembrane proteins of the inner nuclear membrane and interact with the nuclear lamina. The link to the cytoplasm is established by a nesprin transmembrane domain in the outer nuclear membrane and an amino-terminal region projecting into the cytoplasm. The amino-terminal regions of the four nesprins differ from each other, and numerous splice variants exist for each nesprin gene. They encode a varying number of spectrin repeats, besides calponin homology domains and other sequence features. The calponin homology domains are involved in binding nesprins to the actin network (Starr and Han 2002; Zhen et al. 2002; Padmakumar et al. 2004). Moreover, the protein nesprin 3 forms a connection to the intermediate filament network, by

binding to the linker protein plectin (Wilhelmsen et al. 2005). Finally, interactions between microtubule-dependent motors and nesprins or nesprin-related KASH proteins have been documented in a variety of experimental systems, including muscular and nonmuscular cell types from vertebrates as well as *Caenorhabditis elegans* (Malone et al. 2003; Meyerzon et al. 2009; Roux et al. 2009; Zhang et al. 2009b; Zhou et al. 2009; Fridolfsson et al. 2010; Yu et al. 2011; Wilson and Holzbaur 2012). A possible mechanism for nuclear positioning may involve nesprins, binding directly or indirectly to microtubule motor proteins that drive the translocation of nuclei along longitudinal arrays of microtubules along the muscle fiber. Interestingly, synaptic nuclei accumulate significantly more nesprin 1 on the nuclear envelope than extra-synaptic nuclei (Apel et al. 2000), raising the possibility that their clustering and retention at the neuromuscular junction requires increased interactions between the nuclear envelope and microtubule motor proteins. While it is unknown whether defects in the microtubule network or defects in microtubule-dependent transport may be causally involved in the pathogenesis of muscular diseases, it is clear that mutations in nesprin-encoding genes correlate with different myopathies, such as Emery-Dreifuss muscular dystrophy or autosomal recessive arthrogryposis (Zhang et al. 2007b; Wheeler et al. 2007; Attali et al. 2009).

2.8 Conclusion

Microtubule organization in differentiating cell types is generally characterized by a loss of microtubule anchoring to the centrosomal surface and by cell type-specific remodeling of the microtubule network from various cytoplasmic and cortical sites. It is unclear how this loss of centrosomal anchoring is regulated. It is possible that upon differentiation, centrosomes maintain microtubule nucleation activity, but that new microtubules are no longer firmly anchored to the pericentriolar material or that they are actively disconnected from the centrosome by severing enzymes. The resulting free minus ends may then permit translocation of these microtubules to novel sites or may lead to increased polymer turnover and disappearance or remodeling. Likely, proteins of the pericentriolar material may be lost from centrosomes in various differentiating cell types, such as seen in muscle cells, and as a consequence, centrosomal microtubule nucleation may be lost with time. Moreover, it remains so far largely unknown how novel non-centrosomal microtubule organization centers form. Strikingly, proteins that are part of the pericentriolar material in undifferentiated cells, such as ninein, are now found enriched at new microtubule-organizing centers, without centrioles being present. Identifying mechanisms that lead to this relocalization and identifying "receptor" proteins for the pericentriolar material at the sites of non-centrosomal microtubule organization will be a challenge for future research activities. Finally, the notion of a "centrosomal protein" will need to change: since proteins of the pericentriolar material can equally be found at non-centrosomal locations upon differentiation, they should be considered "microtubule-organizing proteins" in a broader sense.

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Organizational Properties of the Pericentriolar Material

3

David Comartin and Laurence Pelletier

Abstract

The centrosome is the major microtubule-organizing centre of animal cells. It participates in a number of crucial cellular functions including cell motility, intracellular transport, mitotic spindle assembly/positioning and cilia formation. Centrosome is composed of pair of ninefold symmetric centrioles surrounded by pericentriolar material, or PCM. PCM organization undergoes a series of dramatic changes in its organization and function as cells progress through the cell cycle. Indeed, the rather small interphase centrosome increases dramatically in size and microtubule nucleation capacity from interphase to mitosis, a process referred to as centrosome maturation. Until very recently, the PCM was thought to be largely amorphous. However, it has been elegantly demonstrated in several super-resolution studies that the PCM is highly organized and that the higher-order organizational properties are conserved from flies to humans. In this book chapter, we review current knowledge on the organization and composition of PCM in both interphase and mitosis and discuss how the centrosome landscape is altered through post-translational modifications, mainly mitotic phosphorylation, during centrosome maturation.

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3.1 The PCM in Interphase

3.1.1 Organization of the Interphase PCM

One of the primary functions of a centriole is to serve as an anchor or docking site for a large collection of proteins collectively called the pericentriolar material (PCM). In early electron microscopy (EM) studies, the PCM appeared as a dark electron-dense cloud from which microtubules originated (Gould and Borisy 1977; Telzer and Rosenbaum 1979). It followed that one of the key roles of the PCM, in interphase but more so in mitosis, was to organize the machinery necessary to nucleate and anchor microtubules (Gould and Borisy 1977). In this chapter, we will focus on the molecular architecture of the PCM, key proteins within the PCM and the regulation of the PCM in both interphase and mitosis.

In recent years, a highly detailed structure of the centriole and its cartwheel has emerged (Guichard et al. 2013; Kitagawa et al. 2011; van Breugel et al. 2011). In particular, crystallographic studies of the Sas-6 protein from *Chlamydomonas reinhardtii* and *Caenorhabditis elegans* have revealed the molecular basis of the conserved ninefold symmetry of centrioles, and cryotomographic studies of the centriole from *Trichonympha* have provided highly detailed 3D maps of the cartwheel, microtubule triplets and the linkages among them (Guichard et al. 2013; Kitagawa et al. 2011; Leidel and Gönczy 2003; van Breugel et al. 2011). Similarly, x-ray crystallography is being used to begin to address the intermolecular organization of key centriole structural and duplication proteins, including the STIL-CENPJ interaction and the dimerization and binding of PLK4 to CEP192 (see Table 3.1 for non-human homologues of proteins discussed throughout this chapter) (Cottee et al. 2013; Hatzopoulos et al. 2013; Shimanovskaya et al. 2014). These studies and the approaches used therein promise to yield a complete molecular architecture of the centriole and a deeper understanding of its assembly in the coming years (Gönczy 2012).

Immunofluorescence microscopy (IFM) has been central to advancing the field of centrosome biology. Unlike EM, which cannot distinguish individual proteins in a large electron-dense assembly, IFM allows comparative localization of known proteins within a single cell. This technique has allowed identification of several proteins contained within the PCM. Unfortunately, until recently, the distribution and orientation of molecules within the PCM cloud remained unknown. According to Abbe's Law, wide-field microscopy is limited in axial resolution to approximately half the wavelength of the emitted light ($d = \lambda/2\text{NA}$, where NA is the lens' numerical aperture and λ the wavelength of the light), and the emission wavelength for conventional fluorophores ranges from 450 to 800 nm (for reviews, see Huang et al. 2009, 2010; Yamanaka et al. 2014). In practical terms, any single-molecule fluorescing at 525 nm (i.e. FITC) would appear to be an airy disc of diameter ~230 nm in an ideal microscope (N.A. 1.4 objective) (Huang et al. 2009, 2010; Yamanaka et al. 2014). Centrioles are approximately 100 nm in diameter, and the most tightly associated PCM surrounding them therefore appears as a single spot by conventional IFM (see Fig. 3.1). One of the longest-standing questions in

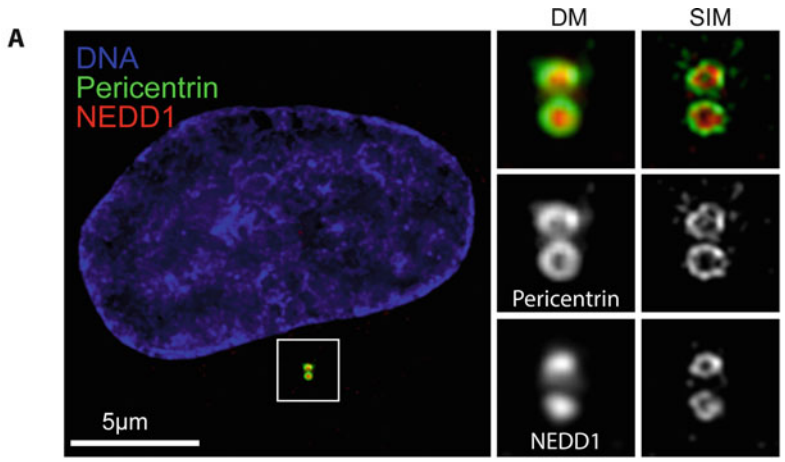
Table 3.1 The HUGO nomenclature

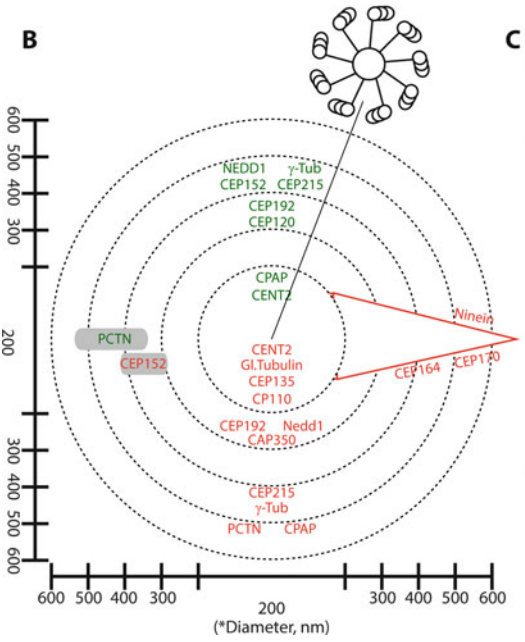
HUGO name	Protein common names/alias (D, *Drosophila*; X, *Xenopus*)
PCNT	Pericentrin, *Drosophila* pericentrin-like protein/D-PLP (D)
AKAP9	A-kinase-anchoring protein, AKAP450, GC-NAP, AKAP350
CDK5RAP2	CDK5 regulatory subunit-associated protein 2, CEP215, centrosomin/CNN (D)
CENPJ	CPAP, centrosomal P4.1-associated protein, SAS-4 (D)
CEP152	Asterless/ASL (D)
NIN	Ninein
PCM1	Pericentriolar material protein 1/PCM-1
CNTROB	Centrosomal BRCA2-interacting protein/centrobin, NIP2
CEP192	SPD-2 (D)
PLK1	Polo-like kinase 1/Plk1, Polo (D), Plx1 (X)
STIL	SCL/TAL1 interrupting locus
NINL	Ninein-like protein
NEDD1	Neural precursor cell expressed developmentally downregulated protein 1, GCP-WD, Dgrip71WD (D)
AURKA	Aurora kinase A
KIZ	Kizuna
SSX2IP	Synovial sarcoma X breakpoint 2 interacting protein
DCTN1	Dynactin 1, $p150^{Glued}$ (D)
TUBG1	γ-tubulin
ODF2	Outer dense fibre of sperm tails 2/hCenexin1

HUGO Gene Nomenclature Committee, http://www.genenames.org, was used for proteins discussed in depth throughout this chapter
Above is a list of commonly used names and alias for each HUGO entry

centrosome biology was whether or not the PCM was organized. Was it specifically arranged, polarized and/or containing discrete domains, or was it an amorphous cloud of interacting proteins glued to the centrioles? There had been some indications that the PCM formed a scaffold and was organized around centrioles as a 'ring' when viewed down the barrel (Dictenberg et al. 1998; Ou et al. 2004). As will be discussed below, the fact that PCM components show defined recruitment dependencies and regulation suggested ordered assembly. But the limitations of conventional microscopy prevented further dissection of potential organization.

Advances in microscopy now allow IFM to surpass (or at least bypass) the resolution limits of conventional microscopy (Huang et al. 2009, 2010; Schermelleh et al. 2010; Yamanaka et al. 2014). These 'super-resolution' microscopy methods include patterned light methods such as three-dimensional structured illumination (3D-SIM) and stimulated emission depletion microscopy (STED) and single-molecule excitation methods such as stochastic optical reconstruction microscopy (STORM) and photoactivated localization microscopy (PALM) (Huang et al. 2009). Of the various methods in use today, 3D-SIM is probably the best commercialized system for being highly amenable to multicolour fluorescence microscopy, while cutting the resolution limits of IFM in half (Gustafsson 2000;





C

Protein	Lawo *et al.*, 2012	Sonnen *et al.*, 2012
CENT2	197	
CP110		165
Gl. Tubulin		166
CEP135		170
CPAP	194	
CEP120	364	
CEP192	395-407	281(m) / 217 (d)
CEP152	435	309-414
Cap350		313 (m) / 279 (d)
CEP215	452-508	429
γ-Tub	497-498	433 (m) / 262 (d)
NEDD1	459-463	300 (m) / 217 (d)
CPAP		498
PCNT	379-581	499
CEP164		394
CEP170		540
Ninein		552

Fig. 3.1 Organization of the interphase PCM of human centrosomes. (**a**) Resolution difference between conventional deconvolution microscopy and three-dimensional structured illumination microscopy (3D-SIM). An interphase human cell labelled with DAPI (nucleus) and fluorescent antibodies against pericentrin and Nedd1 is shown (*left panel*). The *middle* and *right panels* show fourfold magnifications of the outlined centrosome region in cell as imaged in either deconvolution (DM) or 3D-SIM (SIM) microscopy. Note that Nedd1 rings become resolved only when imaged using 3D-SIM. (**b**) Diagrammatic representation of the PCM region surrounding a centriole as a series of regions, indicated by *dashed circles*, each with an incremental increase of 100 nm, in diameter beginning with 200 nm. Rings and diameter guides (*left, bottom*) are drawn to scale. Protein names are given within the region they have been reported to localize to (Lawo et al. 2012; Sonnen et al. 2012). PCTN and CEP152 have been reported to span larger regions of the PCM, as indicated by their *grey boxes*. Protein locations in *green* are from Lawo et al. (2012), and those in *red* are from Sonnen et al. (2012), with the *red triangle* indicating

Huang et al. 2009, 2010; Yamanaka et al. 2014). This technological innovation allows successful probing of the interphase PCM organization. In 2012, parallel studies (of human and fruit fly interphase centrosomes) utilized super-resolution IFM and antibodies targeting known epitopes within select PCM proteins (Fu and Glover 2012; Lawo et al. 2012; Mennella et al. 2012; Sonnen et al. 2012). PCNT (D-PLP in fruit flies; see below) was found to adopt an elongated conformation spanning ~ 200 nm, with the amino-terminal portion (hereafter N or NTD for amino-terminal domain) of the protein aimed away from the centrioles (Fu and Glover 2012; Lawo et al. 2012; Mennella et al. 2012; Sonnen et al. 2012). In humans, CDK5RAP2 and CEP152 appeared polarized with regard to the centrioles, although over shorter spans (Lawo et al. 2012; Sonnen et al. 2012). The other PCM proteins examined in these studies (CEP192, CENPJ, CEP120 and NEDD1 (human)) each occupied distinct radii around the centrioles, as indicated by similar localizations of antibodies targeting opposite ends of each protein (Lawo et al. 2012; Mennella et al. 2012; Sonnen et al. 2012). In G1, about half of cells have only one centriole with a complete shell of PCM, but by G2 both mother centrioles are surrounded by PCM rings with small gaps corresponding to the sites where daughter procentrioles have assembled (Lawo et al. 2012; Mennella et al. 2012). From this work a picture emerged of the interphase PCM as a network of proteins organized in discrete rings by large scaffold proteins. A complete schematic of the human PCM proteins was measured, and their reported localizations are shown in Fig. 3.1.

3.1.2 The PCM at the Centriole Wall

In addition to its function in centriole duplication, CENPJ (Sas-4 in *Drosophila*) may have an important role in PCM recruitment (Gopalakrishnan et al. 2011; Kirkham et al. 2003; Leidel and Gönczy 2003; Pelletier et al. 2006). CENPJ localizes to the centriole barrel, but may also have a distinct population within the outer portion of the PCM (Kleylein-Sohn et al. 2007; Lawo et al. 2012; Sonnen et al. 2012). Patient mutations in CENPJ have led to its classification as an MCPH protein (MCPH6) (Bond et al. 2005; Kaindl 2014; Leal et al. 2003). Notably, a CENPJ-interacting protein called STIL is also an MCPH protein (MCPH7) (Arquint and Nigg 2014; Bond et al. 2005; Kaindl 2014; Kraemer et al. 2011; Kumar et al. 2009; Leal et al. 2003). Initially, human CENPJ was identified as a centrosomal P4.1-associated protein (CPAP) interacting with the 4.1R protein and TUBG1 (γ-tubulin) (Hung et al. 2000). Human CENPJ has a number of critical

Fig. 3.1 (continued) appendages (distal and subdistal). (**c**) Table of reported diameters from Lawo et al. (2012) and Sonnen et al. (2012). Note that Lawo et al. (2012) used the outer edge of the protein toroid to determine the diameter, whereas Sonnen et al. (2012) used max intensity to determine the diameters. For proteins where multiple antibodies were used, maximal ranges are given. For some proteins from Sonnen et al. (2012), measurements were made on mother (*m*) and daughter (*d*) centrioles as indicated

interactions with centriole assembly proteins including STIL, SASS6, CEP135, CEP120 and CNTROB, making it a key organizer of centriole assembly proteins and regulator of centriole length (Comartin et al. 2013; Lin et al. 2013a; Tang et al. 2011). Sas-4 in *C. elegans* is also required for centriole duplication, but in cells with partial depletion of Sas-4, defective centrioles organized less PCM (Kirkham et al. 2003). Accordingly, recent evidence suggests that in *Drosophila*, Sas-4 may function in recruitment of PCM components and that Sas-4 is present in the cytoplasm as well as at the centrosomes in vivo (Gopalakrishnan et al. 2011). Sas-4 appears to participate in multiple cytoplasmic complexes, including one called the 'S-CAP' (Sas-4-CNN-ASL-D-PLP) composed of PCM proteins, another that includes the components of the γ-tubulin small complex (γ-TuSC) (Grip84, Grip91) and finally one that includes all the components of the γ-tubulin ring complex (γ-TuRC) (Grip128, Grip163, Grip75, along with Grip84 and Grip91) (Gopalakrishnan et al. 2012, 2011) (more information on γ-tubulin and its role in microtubule nucleation can be found in the Chap. 4 by Sánchez-Huertas, Freixo and Lüders).

When Sas-4 is overexpressed, acentriolar cytoplasmic foci form that include ASL and D-PLP, and cytoplasmic portions of sucrose gradients from these cells show a unique set of fractions that contain Sas-4, CNN, ASL and CP-190 that are not observed in control cells (Gopalakrishnan et al. 2011). This suggests that Sas-4 can recruit CNN, D-PLP, ASL and CP-190 to cytoplasmic foci or aggregates (Gopalakrishnan et al. 2011). The authors further show that deleting the tubulin-binding PN2-3 domain of Sas-4 removes the interactions with CNN, D-PLP and ASL but not CP-190 or TUBG1 (Gopalakrishnan et al. 2011). Most convincingly, when purified salt-stripped centrosomes (lacking CNN, ASL, CP-190 and Sas-4) are combined with a purified recombinant fragment of Sas-4 (missing the first 90 amino acids) and recombinant CNN and ASL, both CNN and ASL along with the Sas-4 fragment become bound to salt-stripped centrosomes (Gopalakrishnan et al. 2012). When CNN and ASL alone are mixed with these centrosomes, this effect is not observed, indicating that the Sas-4 fragments are tethering CNN and ASL to the naked centrioles in vitro (Gopalakrishnan et al. 2011). Thus, in *Drosophila* at least, Sas-4 can 'deliver' CNN (CDK5RAP2) and ASL (Cep152) to centrioles independent of a role in centriole assembly.

Fine-mapping and crystallographic studies of the interactions between CENPJ and a variety of interacting proteins provide the basis for two recent predictions of the PCM organization immediately surrounding centrioles (Hatzopoulos et al. 2013; Leidel and Gönczy 2003; Lin et al. 2013a). Both models place the CENPJ CTD alongside CEP135 and the NTD along the centriolar microtubules, with one model predicting CENPJ NTD arranged parallel to the cartwheel spokes, the other model predicting CENPJ oriented parallel to the triplet microtubule (Hatzopoulos et al. 2013; Lin et al. 2013a). STIL forms a complex with CENPJ and hsSAS-6 and is required for their localization to procentrioles (Tang et al. 2011). Recent evidence suggests that RBM14 competitively binds STIL to prevent premature STIL-CENPJ interaction and block ectopic centriole assembly (Shiratsuchi et al. 2014). The crystal structures of the CENPJ conserved glycine-

rich CTD (called the G-box or TCP) from *Danio rerio* have been reported, along with co-crystal structures with the CENPJ-interacting domain of STIL (Cottee et al. 2013; Hatzopoulos et al. 2013). The G-box of CENPJ forms an extended β-sheet that can multimerize into long fibrils in vitro and act as binding sites for a conserved domain within STIL (Cottee et al. 2013; Hatzopoulos et al. 2013). These studies led to the suggestion that CENPJ forms polymers along the centriolar axis through its G-box domain, which would be consistent with the predicted interaction of CEP135 with the CTD of CENPJ (Hatzopoulos et al. 2013; Lin et al. 2013a).

CEP120 is another CENPJ-interacting centriole-associated protein that binds microtubules (Comartin et al. 2013; Lin et al. 2013b; Mahjoub et al. 2010; Xie et al. 2007). CEP120 occupies a domain very near the centriole barrel in both EM and super-resolution IFM studies (Comartin et al. 2013; Lawo et al. 2012; Mahjoub et al. 2010). CEP120 interacts with microtubules via its NTD (residues 1–209), but also dimerizes and localizes to the centrosome via its C-terminal coiled-coil domain (731–986 and 700–988, respectively) and interacts with CENPJ via a domain between them (residues 416–730) (Lin et al. 2013b; Mahjoub et al. 2010). Again, the C-terminal domain of CENPJ (residues 895–1070) mediates the binding of CEP120 to this protein (Lin et al. 2013b). Structurally, this suggests that CEP120 dimers are located with their CTD near CENPJ/CEP135, although the position and orientation of its N-terminal microtubule-binding domain are not clear. Functionally, CEP120 cooperates with CENPJ to drive centriole elongation when overexpressed (Comartin et al. 2013; Lin et al. 2013b). Consistent with the shared function in centriole elongation, CENPJ-induced centriole elongation is also blocked by depletion of CEP120 (Comartin et al. 2013; Lin et al. 2013b). CEP120 preferentially localizes to procentrioles, and a proportion of CEP120 undergoes exchange between the cytoplasm and centrioles (Mahjoub et al. 2010). The similarity in functions of CEP120 and CENPJ, as well as their direct interaction, raises the intriguing but untested possibility that CEP120 might also help deliver and tether other PCM components to the centrioles.

A second procentriole-enriched protein called CNTROB (centrosomal BRCA2-interacting protein) was identified in 2005, and like the other CENPJ-interacting proteins, CNTROB is required for centriole duplication (Zou 2005). Like CENPJ and CEP120, CNTROB binds tubulin in vivo and in vitro, through its C-terminal region (residues 765–903) (Gudi et al. 2011). When overexpressed, the tubulin-binding domain of CNTROB displaces native CNTROB from centrioles and destabilizes the existing centrioles (Gudi et al. 2011). CNTROB interacts with CEP152, and RNAi of CNTROB blocks CENPJ recruitment to centrosomes but not vice versa (Gudi et al. 2011, 2014). Like CEP120, CNTROB is required for CENPJ-induced centriole elongation, as pre-depletion of CNTROB prevents overexpressed CENPJ from localizing to centrosomes (Gudi et al. 2014). Remarkably, when either the CENPJ-binding or CEP152-binding domains of CNTROB (residues 183–364 and 1–364, respectively) are overexpressed, CENPJ is removed from both mother and daughter centrioles, consistent with either competitive binding of CENPJ under dynamic exchange or competitive displacement of a complex of CNTROB-CENPJ from centrioles via competition at the CEP152

binding site (Gudi et al. 2014). Regardless, the fact that CNTROB is required for CENPJ localization and retention at centrioles suggests that CNTROB may also share a role in PCM recruitment and anchoring at centrosomes (Gudi et al. 2014).

CEP152 is required for CDK5RAP2, CNTROB and CENPJ recruitment to centrosomes, and CEP152 may cooperate with CEP192 to recruit the kinase PLK4 (Firat-Karalar et al. 2014; Guernsey et al. 2010; Habedanck et al. 2005; Kaindl 2014; Kim et al. 2013; Kleylein-Sohn et al. 2007; Sonnen et al. 2013). CEP152 depletion results in over-accumulation of PLK4, whereas co-depletion of CEP192 and CEP152 results in loss of PLK4 from centrosomes to a degree greater than CEP192 depletion alone (Kim et al. 2013; Sonnen et al. 2013). CEP192 interacts with CEP152 and appears to be partially required for the localization of CEP152 and PLK4 to the centrosome (Sonnen et al. 2013). Finally, the orientation of CEP152 within the PCM is similar to PCNT (CTD near the centriole, NTD extended further into the PCM), suggesting that it adopts an extended conformation (Sonnen et al. 2012).

3.1.3 Large Scaffolds of the Interphase PCM

One of the most important and best studied PCM proteins is PCNT, a coiled-coil domain-containing protein with two isoforms (~378 kDa and ~356 kDa) that localizes to the centrosome (Delaval and Doxsey 2010; Doxsey et al. 1994). PCNT forms the most elongated scaffold yet found in the interphase PCM, as detected by the differences in localization of antibodies targeting epitope sequences within the N-terminal or carboxy-terminal (hereafter C or CTD) portions of PCNT (Lawo et al. 2012; Mennella et al. 2012). Many proteins have been identified as PCNT-interacting partners (reviewed in Delaval and Doxsey (2010)). The list of proteins includes PLK1, PCM1, DISC1, Chk1, PKA, PCKβII, BCR-ABL, IFT, PC2, NEK2, AKAP-450, CDK5RAP2, calmodulin and the γ-TuRC components TUBG1 and GCP2 and GCP3 (see Delaval and Doxsey 2010; Lee and Rhee 2011; Li et al. 2001; Zimmerman et al. 2004). PCNT has been implicated in a number of diseases (Delaval and Doxsey 2010). Chiefly, mutations in the PCNT gene are associated with Majewski microcephalic osteodysplastic primordial dwarfism type 2 (MOPDII) and Seckel syndrome (Griffith et al. 2008; Rauch et al. 2008). Additionally, PCNT is implicated in multiple psychiatric disorders through its requirement for the localization of DISC1 (disrupted in schizophrenia gene 1) to the centrosomes (Delaval and Doxsey 2010; Miyoshi et al. 2004). Recent work has revealed a novel role for PCNT as a possible negative regulator of microtubule nucleation from interphase centrosomes (Lerit and Rusan 2013; O'Rourke et al. 2014). In human interphase cells depleted of PCNT, the number of microtubules grown from centrosomes following cold shock is increased, and in interphase *Drosophila* neuroblasts (NBs), the inactive centrosome has higher levels of D-PLP (Lerit and Rusan 2013; O'Rourke et al. 2014). Depletion of either CEP192 or PCNT leads to increased centrosomal levels of the other, suggesting binding site competition or negative regulation (O'Rourke et al. 2014; Zhu

et al. 2008). In interphase NBs, D-PLP negatively correlates with Spd-2, TUBG1 and Polo kinase recruitment to centrosomes, and removal of D-PLP results in localization of TUBG1 and Polo to both centrosomes (Lerit and Rusan 2013). Thus, it is tempting to speculate that in the case of *Drosophila* NBs, the prevention of Polo recruitment might be mediated through D-PLP inhibition of Spd-2 (Cep192) localization in interphase, similar to the negative regulation of CEP192 and microtubule nucleation by PCNT in interphase human cells (Lerit and Rusan 2013; O'Rourke et al. 2014). This recent data also suggests that CEP192 may play a carefully regulated role in interphase centrosome microtubule nucleation (O'Rourke et al. 2014). As will be discussed below, CEP192 is a major regulator of PLK1 and TUBG1 recruitment to mitotic centrosomes, and in stark contrast to interphase, phosphorylated mitotic PCNT appears to be required for CEP192 localization to centrioles in mitosis (Joukov et al. 2014; Lee and Rhee 2011).

AKAP9 (for 'A-kinase-anchoring protein') encodes a coiled-coil PCM protein even larger than PCNT (Schmidt et al. 1999; Takahashi et al. 1999; Witczak et al. 1999). The largest isoform (of 6) has a molecular weight of ~454 kDa (Takahashi et al. 1999; Witczak et al. 1999). Two studies initially described AKAP9 as being localized to the centrosome and Golgi apparatus (CG-NAP comes from centrosome and Golgi-localized PKN-associated protein) (Takahashi et al. 1999; Witczak et al. 1999). Both studies found AKAP9 while searching for interactions of protein kinases (Takahashi et al. 1999; Witczak et al. 1999). AKAP9 has since been shown to interact with protein kinase A (PKA) type II regulatory subunit RIIα, as well as PKN, CK1δ, CK1ε and the protein phosphatases PP2A and PP1 (Keryer et al. 2003b; Sillibourne et al. 2002; Takahashi et al. 1999; Witczak et al. 1999). This implicates AKAP9 as an important centrosomal docking site for regulatory kinases and phosphatases. The AKAP9 protein shares some homology to PCNT, and interestingly the two proteins interact (Takahashi et al. 2002). AKAP9 also requires a protein called CEP72 for proper localization to both interphase and mitotic centrosomes (Oshimori et al. 2009).

The C-terminal region of AKAP9 can competitively displace full-length AKAP9 and can also displace PCNT from the centrosome (Gillingham and Munro 2000; Keryer et al. 2003b). Both AKAP9 and PCNT harbour a PACT domain (PCNT-AKAP9 centrosome targeting) (Gillingham and Munro 2000). PACT domains are critical for both PCNT and AKAP9 localization to PCM and are now regularly used as fusions to force other peptides to localize to the centrosome (discussed below) (Gillingham and Munro 2000). That the C-terminal region of AKAP9 can displace PCNT suggests that the binding sites of PCNT and AKAP9 at centrosomes could be shared and limited and that there is dynamic exchange with cytoplasmic pools of these proteins in interphase (Gillingham and Munro 2000). Interestingly, the interacting partner of the PACT domain at the centrosome that acts to anchor these PCM proteins is unknown, and how PCNT interacts with the centriolar wall is an important open question (Leidel and Gönczy 2003). The structure of AKAP9 within the PCM is also unknown, as it was not included in recent super-resolution mapping studies of PCM organization. However, it is tempting to speculate that it

might orient itself similar to PCNT given their shared PACT domains and adopt a similarly extended conformation.

The third large coiled-coil scaffold of the PCM is CDK5RAP2/CEP215/CNN. CDK5RAP2 was identified as an interacting partner of CDK5 regulating kinase I (Nagase et al. 2000). The longest of the four splice variants is 215 kDa, and it contains multiple domains, including two 'CNN motif' domains termed CM1 and CM2, as well as two SMC (structural maintenance of chromosomes) domains (Kraemer et al. 2011; Wang et al. 2010). The CM1 domain is involved in the interactions with γ-TuRCs, and the CM2 domain is involved in interactions with PCNT, the Golgi network and calmodulin (Fong et al. 2008; Kraemer et al. 2011; Wang et al. 2010). In addition to these domains, EB1 and CDK5 regulatory kinase I interaction domains have been identified within other regions of the protein (Ching et al. 2000; Fong et al. 2009; Kraemer et al. 2011). Like PCNT and AKAP9, CDK5RAP2 is a coiled-coil domain-containing protein with a variety of binding partners and cellular function. Like AKAP9, CDK5RAP2 is localized to both the Golgi (a non-centrosomal microtubule-organizing organelle) and the centrosome (Rivero et al. 2009; Wang et al. 2010). CDK5RAP2 co-immunoprecipitates (co-IPs) with PCNT and AKAP9 as a complex (Kraemer et al. 2011; Wang et al. 2010). When either PCNT or CDK5RAP2 is overexpressed, both proteins are enriched around interphase centrosomes (Fong et al. 2008; Lawo et al. 2012). In addition to interactions with PCNT and AKAP9, CDK5RAP2 also interacts with CEP152 (discussed later) and is dependent on CEP152 for its localization to the PCM (Firat-Karalar et al. 2014). Depletion of CEP192 also leads to reduction of CDK5RAP2 at interphase centrosomes, suggesting that CEP192 may also be important for CDK5RAP2 localization (O'Rourke et al. 2014). CDK5RAP2 mutations have been identified in patients with primary autosomal recessive microcephaly (MCPH), and CDK5RAP2 is also referred to as MCPH3 (Bond et al. 2005; Kaindl 2014; Kraemer et al. 2011; Moynihan et al. 2000). Consistent with their roles in microcephaly, the loss of CDK5RAP2 or PCNT leads to depletion of neural progenitor cells in mouse embryos (Buchman et al. 2010).

3.1.4 Regulation of Interphase PCM Assembly

The size of the interphase PCM at *Drosophila* centrosomes appears to be controlled by the rate of incorporation of CNN (the CDK5RAP2 homologue) (Conduit et al. 2010). In CNN-null flies, GFP-CNN incorporation into the PCM is faster when two copies of the gene are introduced than when a single copy is present (Conduit et al. 2010). Along with faster incorporation, the total amount of PCM is also increased when two copies of the gene are present (Conduit et al. 2010). CNN appears to first localize to the wall of the centriole and then to migrate into the peripheral PCM (Conduit et al. 2010). Two-channel live-imaging experiments show that GFP-CNN recovers first at the centrioles and then the protein migrates outwards (Conduit et al. 2010). CNN interacts with DSpd-2(CEP192), ASL (CEP152), D-PLP (PCNT) and DSas-4 (CENPJ) by co-IP. Of these, ASL and

DSpd-2 are the strongest candidates for centriolar docking sites for CNN, since injection of ASL or DSpd-2 antibodies drastically reduced GFP-CNN recruitment compared to D-PLP or DSas-4 antibodies (Conduit et al. 2010). CDK5RAP2 may also play a key role in human interphase PCM size regulation, as overexpression of either CDK5RAP2 or PCNT in interphase results in an expansion of PCM beyond typical interphase rings that is reminiscent of mitosis and enrichment of both proteins along with TUBG1 and NEDD1 around the centrosome (Lawo et al. 2012). Notably, CEP192 does not get additionally recruited by CDK5RAP2 or PCNT overexpression, nor does CEP192 overexpression cause enlargement of interphase PCM (Lawo et al. 2012). As will be discussed below, CEP192 is carefully regulated and critical for mitotic PCM function.

Gopalakrishnan and colleagues show that the nucleotide binding state of the α/β tubulin dimers bound to Sas-4 played a critical regulatory role in delivery of PCM components to centrosomes. When a Sas-4 mutant that cannot bind tubulin was expressed in cells, centrosomes became larger and contained additional CNN (Gopalakrishnan et al. 2012). β-tubulin is a GTPase; in the cytoplasm, the predominant form of β-tubulin is GTP-bound tubulin (David-Pfeuty et al. 1977; Desai and Mitchison 1997; Kobayashi 1975). When centrosome-free cytoplasmic extracts were incubated with GDP before immunoprecipitation with Sas-4 antibodies, the amounts of other proteins in complex with Sas-4 increased, whereas incubation with the non-hydrolysable GTP analogue (GMPCPP) drastically destabilized these complexes (Gopalakrishnan et al. 2012). The NTD (amino acids 1–190) of Sas-4 is able to act as a GTPase-activating protein (GAP) for tubulin, catalysing the hydrolysis of GTP into GDP and remaining tightly bound to GDP-tubulin (Gopalakrishnan et al. 2012). Incubation of Sas-4 complexes with GMPCPP and centrosomes results in delivery and stable association of CNN and ASL with centrosomes, but release of Sas-4 and tubulin (Gopalakrishnan et al. 2012). Sas-4 encounters GDP-tubulin at the G2/M transition, causing it to form stable S-CAP complexes for delivery to the centrosome which acts like a guanine nucleotide exchange factor for tubulin, leading to the release of Sas-4 from the centrosome where the key PCM cargo is retained (Gopalakrishnan et al. 2011, 2012). Therefore, the PN2-3 domain of Sas-4/CENPJ might function as a regulatory domain for Sas-4, but when overexpressed, it can bind GTP-tubulin dimers and catalyse hydrolysis to create GDP-tubulin, depleting the pool of available tubulin for polymerization. Consistent with this, highly overexpressed CENPJ forms aggregates that contain tubulin, but not microtubule polymers (Hsu et al. 2008).

3.1.5 Interphase Microtubule Nucleation and Anchoring in the PCM

In many cell types, the interphase centrosomes organize the microtubule cytoskeleton. Microtubules are nucleated from a complex called the γ-tubulin ring complex (γ-TuRC) as described elsewhere in this book. Several PCM proteins have been shown to bind γ-TuRCs or TUBG1 and to be important for the interphase

microtubule cytoskeleton. NIN is a 245 kDa protein containing multiple coiled coils, a guanine nucleotide binding site, an EF-hand and four leucine zipper domains (Bouckson-Castaing et al. 1996). Mutations in NIN (Ninein) have been identified in microcephalic primordial dwarfism (MPD) (Dauber et al. 2012). By immuno-EM, NIN localizes to the PCM at sites of microtubule nucleation, as well as the subdistal appendages of the mother centriole and the proximal ends of both centrioles (Bouckson-Castaing et al. 1996; Mogensen et al. 2000). Overexpressed NIN localizes to the PCM and forms an extended focus that strongly recruits γ-TuRCs and dynein-DCTN1 (DCTN1) (Casenghi 2005). Also, NIN co-immunoprecipitates with TUBG1 and several dynactin subunits (Casenghi 2005; Delgehyr 2005). The proper recruitment of NIN requires DCTN1, and both DCTN1 and NIN are dependent on Kif3a for their localization, suggesting that Kif3a is an upstream recruitment factor important for the assembly of mother centriole appendages (Kodani et al. 2013). NIN may be recruited to the centrosome via centriolar satellites, which are dynamic macromolecular complexes that include multiple proteins important for centrosome and cilia assembly and function (Bärenz et al. 2011; Kubo 2003; Kubo et al. 1999; Löffler et al. 2012; Prosser et al. 2009; Tollenaere et al. 2015). Loss of NIN from centrosomes results in loss of interphase microtubule organization capability at the centrosome (Dammermann 2002). NIN fragments consisting of the NTD (residues 1–373) and the central coiled-coil region (residues 373–1874) do not localize to the centrosome; however, the CTD (residues 1874–2113) is sufficient for localization to both centrioles equally and apparently displaces the endogenous NIN and TUBG1 from the centrosomes (Delgehyr 2005). When a fusion of the NTD and CTD fragments (omitting residues 373–1874) is expressed, this NIN construct restores preferential labelling of the mother centrioles (Delgehyr 2005). More interestingly, the N + C fusion NIN is capable of facilitating microtubule nucleation at centrosomes, but not retention of microtubules (Delgehyr 2005). This leads to the conclusion that NIN has a dual role at the centrosome, in facilitating microtubule nucleation by γ-TuRC recruitment through its NTD and then in maintaining anchorage of the microtubules at the centrosomes (Delgehyr 2005). Although the regulation of NIN is not well studied, the N-terminal portion of NIN is conserved within another protein, NINL (Ninein-like protein).

NINL was identified in a yeast two-hybrid screen for PLK1 targets (Casenghi et al. 2003). It shares 37 % identity between its N-terminal half and the N-terminal end of NIN, a region that includes the EF-hand domain, though the C-terminal portions of each protein are unrelated besides having coiled coils (Casenghi et al. 2003). NINL is found to localize to interphase centrosomes and by co-IP to interact with PLK1, TUBG1 and GCP4 (Casenghi et al. 2003). When overexpressed, large assemblies of NINL form at the centrosome that are capable of nucleating microtubule asters following release from cold treatment in cells, and purified NINL mixed with *Xenopus* egg extracts is capable of nucleating microtubules (Casenghi et al. 2003). The N-terminal portion of NINL, but not the C-terminal portion, is able to nucleate microtubules in the cytoplasm when overexpressed, indicating that the microtubule nucleation activity resides there (Casenghi et al. 2003). Remarkably, co-expression of a constitutively activated

mutant PLK1 (PLK1-T210D) that functionally mimics mitotic PLK1 with NINL leads to fragmentation of these centrosomal assemblies (Casenghi et al. 2003). In U-2 OS cells, PLK1-T210D (constitutively active) expression completely removes native NINL from interphase centrosomes, whereas wild-type PLK1 expression does not (Casenghi et al. 2003). Consistent with this, NINL is lost from centrosomes at the onset of maturation and absent throughout mitosis (Casenghi et al. 2003).

CDK5RAP2 is important for the localization of TUBG1 to the interphase centrosome, and depletion of CDK5RAP2 leads to loss of TUBG1 and failure of microtubule nucleation at centrosomes following nocodazole washout (Fong et al. 2008). The depletion of CDK5RAP2 in interphase does not displace PCNT, consistent with the idea that in interphase PCNT is not a primary TUBG1 binding site at the centrosome (Fong et al. 2008; O'Rourke et al. 2014; Zimmerman et al. 2004). AKAP9 is the other scaffold protein that appears to play a role in interphase microtubule nucleation at the centrosomes (Oshimori et al. 2009; Takahashi et al. 2002). AKAP9 binds γ-TuRC subunits GPC2 and possibly GPC3, although it is unclear which isoform of AKAP9 is required for γ-TuRC localization to centrosomes (Keryer et al. 2003a; Takahashi et al. 2002). When CEP72 is depleted in interphase, AKAP9 is lost from centrosomes, along with a significant proportion of TUBG1 (Oshimori et al. 2009). Centrosomes depleted of CEP72 cannot nucleate microtubule asters following washout of nocodazole (Oshimori et al. 2009). The depletion of AKAP9 also results in centrosomes that cannot nucleate microtubules following nocodazole washout; however, AKAP9 depletion does not remove TUBG1 from these centrosomes (Keryer et al. 2003a; Oshimori et al. 2009). Thus, CEP72 is critical for both TUBG1 and AKAP9 recruitment to interphase PCM, while CDK5RAP2 plays a key role in TUBG1 recruitment, and AKAP9 is important for the microtubule nucleating ability of interphase centrosomes downstream of TUBG1 recruitment (Fong et al. 2008; Oshimori et al. 2009).

3.2 The PCM in Mitosis

3.2.1 Organization of the Mitotic PCM

The demand on mitotic centrosomes to nucleate and anchor microtubules mandates an increase in the size of the PCM leading up to mitosis. There are microtubule nucleation events within the mitotic spindle, and at the chromosomes themselves, but the primary sources of microtubule nucleation for the mitotic spindle are the centrosomes (Lüders and Stearns 2007; Piehl et al. 2004) (for details see the Chap. 1 by Meunier and Vernos). Centrosome maturation refers to the process wherein the PCM surrounding centrioles expands at the onset of mitosis to facilitate increased microtubule nucleation and anchoring (Piehl et al. 2004). This process is carefully regulated, and many PCM proteins have been shown to be critical for proper

recruitment or anchoring of the γ-TuRCs, and consequently spindle microtubules, in mitosis.

The proteins of the interphase PCM are organized within distinct domains around the centrioles (Fu and Glover 2012; Lawo et al. 2012; Mennella et al. 2012; Sonnen et al. 2012). In mitosis, although the expanded PCM no longer forms discrete rings, careful correlation analyses indicate that there are persistent patterns of organization among the proteins (Lawo et al. 2012). These organization patterns persist even when the PCM is fragmented artificially by depletion of HAUS6 or microtubule nucleation factors or when cells are treated with nocodazole to depolymerize microtubules (Lawo et al. 2009; 2012). Thus, like interphase PCM, mitotic PCM is highly organized spatially, and this organization is maintained even without microtubules. In the following section, we describe the roles of select proteins in mitotic PCM assembly, as well as the regulation of centrosome maturation by multiple kinases, most notably PLK1. The kinases phosphorylating key PCM proteins, and the recruitment dependencies of key PCM proteins, are summarized in Table 3.2.

3.2.2 The Regulation of Mitotic PCM Expansion

When the human polo-like kinase 1 (PLK1) was first characterized, it was found to be localized to the centrosomes through interphase and G2 and then focused on the mitotic centrosomes in metaphase, until disappearing from centrosomes and appearing in the central spindle region in anaphase cells and later the midbody through the completion of cytokinesis (Golsteyn et al. 1994, 1995; Lane and Nigg 1996). When antibodies against PLK1 are injected into HeLa cells, centrosome separation, centrosome maturation and ultimately mitotic spindle formation are blocked (Golsteyn et al. 1994, 1995; Lane and Nigg 1996). Consistent with a critical mitotic role, PLK1 reaches peak levels and is activated in mitotically arrested cells (Golsteyn et al. 1994, 1995). PLK1 preferentially binds substrates that have been primed by phosphorylation, notably by Cdk1-cyclin B or NEK2 (Barr et al. 2004; Elia et al. 2003; Elia 2003; Jeong et al. 2007; Rapley et al. 2005; Zitouni et al. 2014). PLK1 interacts with a protein called ODF2 whose depletion results in reduced recruitment of PLK1 and NIN to centrosomes and serious mitotic defects (Soung et al. 2006; 2009). PCM1 is required for both PLK1 and NIN transport to centrosomes, thus it is possible that ODF2 is also involved in satellite assembly or delivery. A variety of PLK1 substrates have been identified in mitosis, both at the centrosome and elsewhere (for reviews, see Barr et al. 2004; Petronczki et al. 2008; Zitouni et al. 2014). Notably, PCM maturation requires PLK1, as depletion of the kinase results in reduced levels of PCNT, CDK5RAP2 and CEP192 at mitotic centrosomes (Haren et al. 2009). PLK1 itself is under cell cycle regulation (reviewed in Zitouni et al. (2014)). Early work in *Xenopus* identified an activating phosphorylation of PLK1 at T201 (human residue threonine 210), and a mutation of T201D creates a constitutively active PLK1 (Qian et al. 1999). It is also known that the PBD domain of PLK1, when expressed

Table 3.2 Known recruitment dependencies and regulatory kinases for PCM proteins discussed within this chapter

Protein	Required for PCM recruitment of	Phosphorylated by	Evidence of γ-TuRC or γ-tub interaction	Required for γ-tub recruitment or anchoring
PCNT	DISC1, CDK5RAP2, CEP192, NEDD1, AURKA,	PLK1	Y	Y
AKAP9	RAN, PKA		Y	Y
CDK5RAP2	DCTN1, PCNT, AKAP9		y	Y
CENPJ	Cnn(*D*),Asl (*D*), D-PLP(*D*)		N	N
CEP72	AKAP9, KIZ		N	Y
CEP152	CDK5RAP2, CNTROB, CENPJ		N	N
KIF3A	NIN, DCTN1		N	N
NIN			Y	Y
PCM1	NIN, PCNT,CETN, PLK1, NEK2, SSX2IP	CDK1, PLK1	N	N
CNTROB	CENPJ	NEK2, PLK1	N	N
CEP192	CEP152, NEDD1, PCNT, AURKA		Y	Y
STIL			N	N
CEP120	SPICE1,CENPJ, CEP135		N	N
PLK1	CEP192, PCNT, CDK5RAP2, ODF2,	AURKA	N	Y
ODF2	NIN, PLK1		N	Y
NEDD1		CDK1, PLK1, NEK9	Y	Y
KIZ		PLK1	Y	N
NINL		CDK1, NEK2, PLK1	Y	N
SSX2IP			Y	Y

See text for references and Table 3.1 for equivalent gene names in other species

alone, can bind the kinase domain of PLK1, except when T210 is mutated to mimic phosphorylation (T210D) (Jang et al. 2002). The interaction of the PBD fragment with PLK1 inhibits the kinase activity of the full-length protein (Jang et al. 2002). This leads to the simple model that PLK1 is auto-inhibitory throughout interphase, but its activation by phosphorylation at T210 corresponds to loss of this auto-inhibition (Jang et al. 2002; Seki et al. 2008b). The mechanism of PLK1's

activation in vivo has since been found to involve the mitotic AURKA kinase and a protein called BORA (Seki et al. 2008b). BORA was identified as giving a mitotic delay when depleted in an siRNA screen of proteins enriched in G2 (Seki et al. 2008a, b). BORA interacts with PLK1 by reciprocal co-IP, and when BORA is depleted, PLK1 phosphorylation at T210 is disrupted (Seki et al. 2008b). Conversely, overexpression of BORA leads to increased PLK1-T210 phosphorylation in asynchronous cells, which is inhibited by treatment with a small-molecule AURKA inhibitor (Seki et al. 2008b). PLK1 kinase phosphorylation at T210 and its activity in vitro are increased by a combination of BORA and AURKA addition, and AURKA depletion in vivo leads to loss of PLK1 phosphorylation at T210 (Seki et al. 2008b). Overall, the model is that PLK1 binds BORA in G2, which induces a conformational change in PLK1 to allow AURKA activation at T210 (Seki et al. 2008b). Once PLK1 becomes activated at T210, it no longer requires BORA, and BORA becomes a target for PLK1 phosphorylation and SCF-β-TrCP degradation to allow the onset of anaphase (Seki et al. 2008a).

NINL is an interphase centrosomal protein that disappears from centrosomes during mitosis. PLK1 phosphorylates the N-terminal half of NINL, which has eight candidate phosphorylation sites (Casenghi et al. 2003). When overexpressed, NINL causes abnormal mitotic spindles, and an NINL-8A mutant with eight potential PLK1 sites mutated to alanines has a more severe effect, consistent with PLK1-mediated removal of NINL before mitosis (Casenghi et al. 2003). The mechanism by which PLK1 phosphorylation of NINL facilitates its removal from centrosomes has been partly elucidated. NINL (and NIN) delivery to centrosomes requires the presence of both a microtubule network and an active dynein-dynactin complex (Casenghi 2005). NIN and NINL share a common N-terminal region which regulates their recruitment and localization to the centrosomes via the activity of the dynein-dynactin microtubule motor complex (Casenghi 2005). Overexpression of NINL recruits large concentrations of dynein-dynactin to the centrosomes, and this is reversible by co-expression of PLK1-T210D, but not by PLK1-K82R or when the NINL-8A mutant is used (Casenghi 2005). Further cementing the negative regulation of NINL-dynactin interaction by PLK1, in vitro phosphorylation of the NINL N-terminal fragment by PLK1 inhibits its interaction with DCTN1 (a dynactin subunit) (Casenghi 2005). Like other mitotic PLK1 substrates, NINL undergoes a priming phosphorylation by NEK2NEK2 (Rapley et al. 2005). When a kinase-dead NEK2 protein is overexpressed, NINL is found to persist in mitotic centrosomes (Rapley et al. 2005). Conversely, overexpression of NEK2 results in displacement of either NINL or the NINL-8A mutant from centrosomes, and NEK2 is able to phosphorylate both forms suggesting NEK2 target sites are distinct from PLK1 target sites (Rapley et al. 2005). Supporting the model of a priming phosphorylation, in vitro phosphorylation assays show that pre-incubation of NINL with active NEK2 leads to a strong increase phosphorylation by PLK1 relative to a kinase-dead NEK2 or buffer (Rapley et al. 2005). NINL is phosphorylated at multiple sites, and in addition to NEK2, CDK1/CCNB also regulates NINL and phosphorylates to prime NINL for PLK1 interaction (Wang and Zhan 2007; Zhao et al. 2010). NINL interacts with CDK1, and the phosphorylation sites for CDK1/

cyclin B were found to be S185 and S589, mutation of both of which results in NINL persisting through mitosis (Zhao et al. 2010). Under normal conditions, NINL interacts with Cdh1 by co-IP and is a target of the Cdh1-APC complex for ubiquitinylation and degradation (Wang and Zhan 2007; Zhao et al. 2010). An NINL S185A/S589A double mutant is stable through mitosis and does not interact with Cdh1 (Zhao et al. 2010). Expression of this variant of NINL causes multinucleate cells, consistent with a mitotic or cytokinetic defect and illustrating the importance of its regulation for mitosis (Zhao et al. 2010). Finally, the S185A mutant NINL does not interact with PLK1, indicating that a phosphorylation event likely primes for the PBD of PLK1 to bind (Zhao et al. 2010).

CEP192 was identified as a centrosomal protein required for proper mitotic spindle formation and centriole duplication (Gomez-Ferreria et al. 2007, p. -; Zhu et al. 2008). CEP192 acts as a scaffold that organizes the mitotic signalling activities of AURKA and PLK1 in both human and *Xenopus* (Joukov et al. 2014). CEP192 is a critical part of PCM maturation, as depletion of CEP192 leads to loss of PCNT, NEDD1, AURKA and TUBG1 at mitotic centrosomes (Gomez-Ferreria et al. 2007; Joukov et al. 2010; Zhu et al. 2008). At the onset of mitosis, CEP192 undergoes an approximately tenfold enrichment on centrosomes, dependent upon PLK1 activity (Gomez-Ferreria et al. 2007; Haren et al. 2009; Zhu et al. 2008). CEP192 interacts with and activates AURKA kinase and is critical for its recruitment to mitotic PCM (Joukov et al. 2010). When AURKA-coated beads are incubated with metaphase *Xenopus* extracts, they co-IP CEP192, Plx1 (*Xenopus* PLK1), NEDD1, and TUBG1, and AURKA becomes activated by trans-autophosphorylation (Joukov et al. 2010, 2014). CEP192 is required for recruitment of all of the proteins listed to AURKA beads and AURKA activation under these conditions (Joukov et al. 2010, 2014). CEP192 interacts with AURKA, leading to AURKA activation, and subsequently AURKA activates Plx1 by phosphorylation (Joukov et al. 2014). Both AURKA and Plx1 can bind and phosphorylate CEP192, and five serine residues were identified as Plx1 phosphorylation-dependent γ-TuRC binding sites (Joukov et al. 2014). When those five serine residues of CEP192 were mutated to alanines, centrosomes and AURKA, coated beads lost their MTOC capability when incubated with metaphase *Xenopus* extracts, consistent with CEP192-mediated γ-TuRC recruitment playing a major role in centrosome maturation (Joukov et al. 2014). Similar to the results in *Xenopus*, CEP192 depletion phenotypes in mitotic HeLa cells were not rescued by a PLK1-binding-deficient CEP192 nor an AURKA-binding-deficient CEP192 construct (Joukov et al. 2014). Finally, CEP192, NEDD1, γ-TuRC, AURKA and PLK1 localization to *Xenopus* sperm centrioles treated with mitotic extracts was dependent on PCNT; however, AURKA beads were able to properly act as MTOCs when treated with metaphase extracts regardless of PCNT being absent (Joukov et al. 2014). Notably, PCNT is also a target of PLK1 phosphorylation, with four different residues identified as PLK1 sites: S1235, S1241, T1209 and T1221 (Lee and Rhee 2011). When endogenous PCNT is depleted, no PCNT is observed at centrosomes, and mitotic spindles do not form properly (Lee and Rhee 2011). Under conditions where endogenous PCNT is depleted, phosphorylation-resistant versions of PCNT (S1235A, S1241A,

T1209A and T1221A) can all localize to the mitotic centrosomes; however, S1235A and S1241A mutants cannot rescue the spindle defects in mitosis (Lee and Rhee 2011). This indicates that these two sites, and their phosphorylation by PLK1, are essential for maturation of functional mitotic centrosomes (Lee and Rhee 2011). In other experiments where endogenous PCNT is removed, the phosphorylation-resistant PCNT proteins are unable to restore mitotic centrosomal localization of CEP192, NEDD1, AURKA and TUBG1, whereas CDK5RAP2 localization to the centrosome appears to depend only on the presence of PCNT, not on its phosphorylation at those sites (Lee and Rhee 2011). Interestingly, in interphase cells PCNT depletion does not affect CEP192/NEDD1/TUBG1 localization (Lee and Rhee 2011). To solidify the importance of PLK1 phosphorylation of PCNT in PCM maturation, Lee and Rhee created a PLK1-PCNT fusion protein and versions of this construct that were kinase dead, constitutively active or phosphorylation resistant (S1235A and S1241A double mutant in PCNT) (Lee and Rhee 2011). In interphase cells, the constitutively active PLK1-PCNT fusion (but not the kinase dead or phosphorylation resistant) drives centrosomes to recruit increased amounts of CEP192, NEDD1 and TUBG1, mimicking the maturation of centrosomes in mitosis (Lee and Rhee 2011). Consistent with CDK5RAP2 recruitment being phosphorylation independent, all three PLK1-PCNT fusions increase CDK5RAP2 localization to centrosomes (Lee and Rhee 2011). Thus, PLK1 phosphorylation of PCNT drives centrosome maturation through increased CEP192 recruitment, where CEP192 then acts as a scaffold for AURKA and PLK1 activation at the centrosome and is phosphorylated by PLK1 leading to a gain in γ-TuRC recruitment that is critical for proper mitotic spindle formation (Joukov et al. 2010, 2014; Lee and Rhee 2011).

In *Drosophila*, mitotic PCM scaffolds are assembled primarily based on two proteins, Spd-2 (CEP192) and CNN (CDK5RAP2). Initially, it was shown that CNN was dynamic at the centrosomes, being recruited first to the centriole wall, then spreading outwards into the PCM (Conduit et al. 2014a, b). Subsequent studies have shown that Spd2 has similar dynamics, and in photobleaching experiments, the Spd2-GFP recovers initially as a toroid around the centriole (Conduit et al. 2014a, b) (see Fig. 3.2). These proteins are the only two PCM components found to be recruited to the centriole first, as GFP fusions to the *Drosophila* homologues of CENPJ, NEDD1, Plk1, AURKA, PCNT, CEP152 and TUBG1 show uniform recovery throughout the PCM following photobleaching (Conduit et al. 2014a, b). ASL appears to be the primary docking site for Spd2 and CNN at the centrioles, as injection of ASL antibodies reduces both Spd2 and CNN recovery rates following photobleaching (Conduit et al. 2010; Conduit et al. 2014a, b). Consistent with this model, simultaneous removal of Spd2 and CNN essentially abolishes PCM maturation in mitosis, but not ASL localization (Conduit et al. 2014a, b). There are subtle but important differences in the PCM scaffolds of CNN and Spd2. First, the mitotic Spd2 scaffold only partially overlaps the CNN scaffold, which reaches farther from the centriole (Conduit et al. 2014a, b). Secondly, the CNN scaffold collapses without microtubules, whereas the Spd2 scaffold appears robustly microtubule independent (Conduit et al. 2014a, b). Finally, using

super-resolution microscopy, a Spd-2 toroid around the centriole is evident within the mitotic scaffold, whereas CNN lacks a strongly defined structure around the centriole (Conduit et al. 2014a, b). Despite these differences, there is clear evidence of a functional relationship. Removal of either protein drastically reduces the overall amount of the other in the PCM (Conduit et al. 2014a, b). Importantly, Spd-2 is able to recover at the centriole wall with similar kinetics regardless of CNN being absent, suggesting that CNN is important for Spd-2 retention/expansion in the mitotic PCM downstream of recruitment by ASL (Conduit et al. 2010; Conduit et al. 2014a, b). Therefore, ASL appears to recruit Spd-2, which recruits CNN, and together they migrate outwards to form a stable mitotic PCM matrix that is required for recruitment of most other mitotic PCM components and the formation of a mitotic spindle (Conduit et al. 2010; Conduit et al. 2014a, b). Together, both proteins appear to be responsible for the vast majority of mitotic PCM assembly (Conduit et al. 2014a, b).

The expansion of *Drosophila* CNN into a mitotic PCM matrix is regulated by Polo kinase (Conduit et al. 2010, 2014a, b). There are ten conserved potential phosphorylation sites within the phospho-regulated multimerization (PReM) domain of CNN, and when all ten are mutated to alanines, CNN loses its ability to expand into a centrosomal scaffold and localizes to the centrosome as what appears to be a centriolar protein (Conduit et al. 2014a, b). Conversely, mutating those ten candidate phosphorylation sites to aspartic acids leads to expanded CNN foci in *Drosophila* embryos, and in vitro the purified protein forms larger complexes based on size exclusion chromatography (Conduit et al. 2014a, b). Although the human CDK5RAP2 has not been identified as a bona fide PLK1 target, it is known that treatment of cells with a PLK1 inhibitor reduces strongly the mitotic accumulation of CDK5RAP2 (and other PCM proteins) (Haren et al. 2009). However, overexpression of CDK5RAP2 in human interphase cells (presumably without active PLK1) does lead to an expansion of a PCM scaffold that strongly incorporates PCNT but weakly incorporates TUBG1 and NEDD1 (Lawo et al. 2012). It will be interesting to see how the regulation of CDK5RAP2 in humans compares to CNN in *Drosophila*.

3.2.3 Expansion of the PCM Matrix During Mitosis

PCNT, CDK5RAP2 and AKAP9 are also important proteins of the expanded PCM shell seen in mitotic cells. AKAP9 further undergoes expansion onto the mitotic spindle, reminiscent of the localization of TUBG1 and CDK5RAP2 (Fong et al. 2008; Kraemer et al. 2011; Takahashi et al. 2002). In terms of recruitment dependencies, a study by Fong et al., in 2008, showed that depletion of CDK5RAP2 did not impact PCNT localization in mitosis (Fong et al. 2008). However, more recent studies using higher resolution indicate that when either CDK5RAP2 or PCNT is depleted, mitotic centrosomes retain an interphase-like ring of the other protein immediately surrounding centrioles, indicating that PCNT and CDK5RAP2 are codependent for expansion into a mitotic PCM matrix (Lawo et al. 2012). As

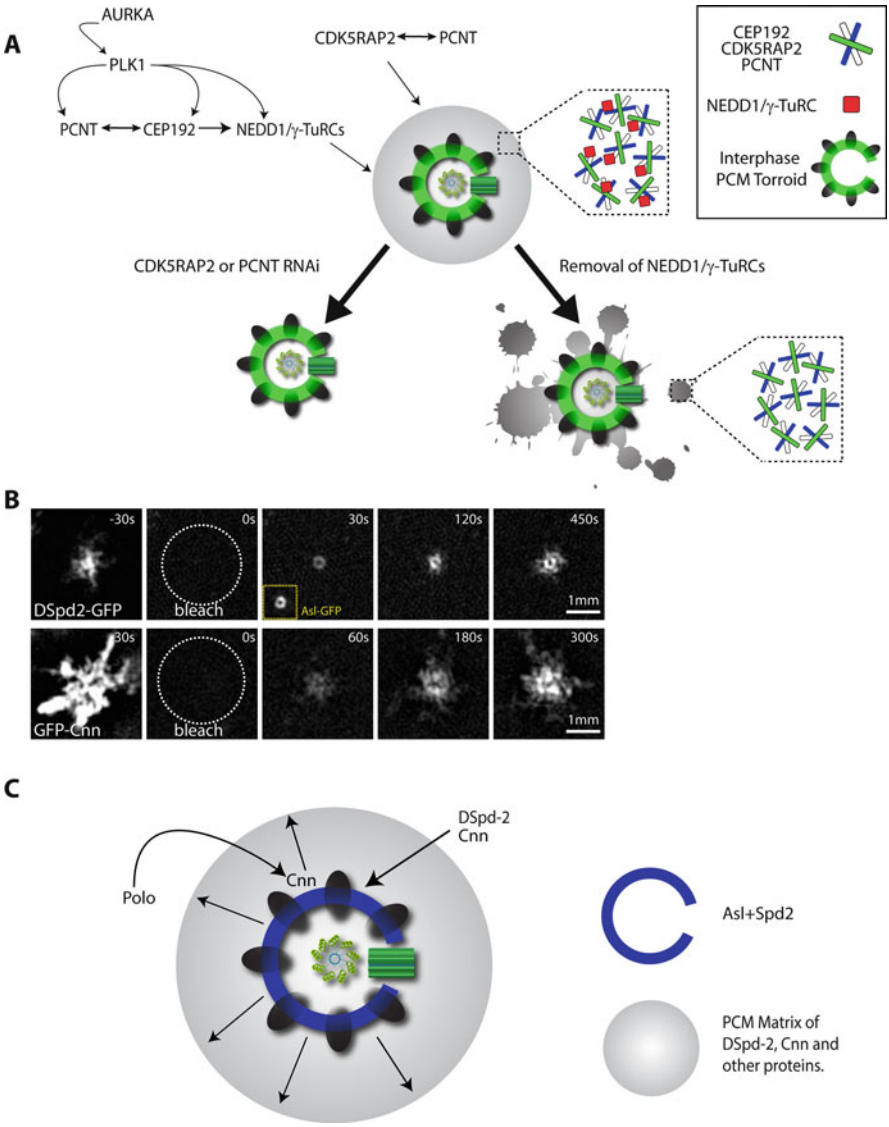


Fig. 3.2 Mitotic PCM in humans and *Drosophila*. (**a**) Pathways contributing to human mitotic PCM expansion. Pericentrin recruits CEP192 which organizes a signalling cascade between AURKA and Plk1 leading to CEP192 phosphorylation which generates g-TuRC binding sites on CEP192 (*left*). CEP192 also recruits NEDD1, which is required for mitotic PCM microtubule nucleation. Curved lines indicate phosphorylation. CDK5RAP2 and pericentrin are codependent for their localization to mitotic PCM. Within the mitotic PCM, distinct organization exists among key proteins. Removal of proteins important for microtubule nucleation lead to fragmentation of the PCM, but despite this fragmentation PCM organization persists, indicating that the assembly and arrangement of the mitotic PCM matrix is microtubule independent in humans. Removal of CDK5RAP2 or PCNT results in loss of the expanded mitotic PCM, but retention on the other protein as a toroid around the centriole, indicating codependence for mitotic PCM matrix expansion. (**b**) Live 3D-SIM FRAP data from Conduit et al. (Conduit et al. 2014a, b) showing the dynamic nature of mitotic PCM recruitment in *Drosophila* (*top*). Note that following

mentioned, overexpression of either PCNT or CDK5RAP2 results in interphase PCM enlargement (Lawo et al. 2012) (see Fig. 3.2). Finally, in keeping with the multifunctional nature of CDK5RAP2, it has been shown to transcriptionally activate the promoters of the checkpoint proteins BUBR1 and Mad2 and binds the APC subunit CDC20, making it an important regulator of the mitotic spindle checkpoint (Zhang et al. 2009a, b). Therefore, AKAP9, PCNT and CDK5RAP2 form the expanded scaffold of the mitotic PCM and play critical roles in mitotic centrosome function.

An important role for mitotic PCM in regulating centrosome inheritance has emerged in recent years. Asymmetric cell division is a process where mitosis creates two distinct daughter cells, one differentiated and one undifferentiated (Morrison and Kimble 2006; Reina and Gonzalez 2014). During such divisions, one of the two centrosomes is often preferentially retained by the non-differentiating stem cell (Morrison and Kimble 2006; Reina and Gonzalez 2014). Differentiation between the two centrosomes is achieved through differences in PCM composition and microtubule nucleating activities between older and younger centrosomes. In *Drosophila* neuroblast stem cells, CNN accumulates on the younger centrosomes prior to asymmetric cell division and is lost from the mother centrioles following a period of decreased incorporation rates (Conduit and Raff 2010). The younger centrosome is anchored to the apical cortex by microtubules, whereas the older centrosome is basically inactive (Januschke et al. 2013). Removal of CNN in the neuroblast niche leads to random retention of mother or daughter centrosomes by the stem cells following mitosis, presumably due to loss of apical cortex anchoring without CNN (Conduit and Raff 2010). Selective retention of CNTROB is also seen on the younger centrosomes in neuroblast cells, and CNTROB localization along with Polo activity is critical for microtubule nucleation by the daughter centrosome (Januschke et al. 2013). Conversely, D-PLP is enriched on the older centrosome in *Drosophila* neuroblast cells, where it appears to prevent unwanted activation of the mother centriole by blocking recruitment of Polo kinase (Lerit and Rusan 2013). Asymmetric cell division is less well studied in mammals; however, evidence to date suggests that the proteins enriched on the more mature mother centriole are critical for centrosome identity in these contexts. In the developing brains of mice, the older centrosomes are retained by glia progenitor cells selectively, and this requires NIN (Wang et al. 2009). NIN is enriched on the more mature mother centriole, and when NIN is absent, cell

Fig. 3.2 (continued) photobleaching (*second panel*), DSpd-2 recovers first as a toroid around the centriole, while CNN also recovers around the centriole first but with less defined structure. Following initial recruitment to the centriole wall, both DSpd-2 and CNN migrate into the larger PCM matrix. Note that DSpd-2 retains a distinct toroid structure around the centriole within the expanded PCM. *Inset in top panel* shows typical ASL toroid for comparison. (**c**) Model of mitotic PCM recruitment and dynamics in *Drosophila*. ASL recruits DSpd-2 to the centriole, which brings with it CNN. CNN is able to expand into a mitotic matrix following phosphorylation by Polo kinase, and the expanded matrix of CNN and DSpd-2 is maintained by both proteins. See text for details (Panel (**b**) is reproduced from Conduit et al. (2014a, b) with permission from the authors)

divisions become symmetrical and progenitor cells are depleted (Ou et al. 2002; Wang et al. 2009). In summary, for stem cell niches, differential recruitment and regulation of mitotic PCM components between older and younger mother centrioles is a critical part of ensuring proper centrosome segregation.

CEP72 is also required for localization of a protein called Kiz (Kizuna, from the Japanese word for 'bonds') that was identified as a PLK1 substrate and interacting protein (Oshimori et al. 2006). Kiz localizes to the centrosomes, but preferentially to the older centrosome, and becomes enriched starting in prophase (Oshimori et al. 2006). Depletion of Kiz results in a striking mitotic phenotype, where multiple foci containing TUBG1, GCP2, PCNT and AKAP9 are observed that are capable of nucleating microtubules (Oshimori et al. 2006). Immediately prior to mitosis, Kiz-depleted cells have two properly matured centrosomes with two centrioles each, but these fragment in prometaphase (Oshimori et al. 2006). This fragmentation is prevented by nocodazole treatment and partially reversed by simultaneous reduction in chromosome pulling forces (Oshimori et al. 2006). Kiz protein levels are elevated in mitosis, and the mitotic protein is phosphorylated by PLK1 (Oshimori et al. 2006). The phosphorylation of Kiz by PLK1 in vitro occurs on Thr 379, and the mitotic phenotypes of Kiz depletion are not rescued by an RNAi-resistant T379A mutant Kiz (Oshimori et al. 2006). Mitotic phenotypes of Kiz depletion, including centrosome fragmentation, are rescued by expression of an RNAi-resistant wild-type Kiz or a Kiz T379E mutant (Oshimori et al. 2006). The PCM stabilization by Kiz may be through interactions with other PCM proteins, since AKAP9, PCNT and TUBG1 co-IP with Kiz (Oshimori et al. 2006). Notably, the Kiz interaction with PCNT is found to be increased in mitosis, and Kiz-T379A only weakly interacts with PCNT whereas Kiz-T379E interacts more strongly than the wild-type protein (Oshimori et al. 2006). Thus, Kiz is an important PLK1-regulated PCM component that interacts with PCNT and is critical for the mitotic centrosome to resist the pulling forces of the mitotic spindle (Oshimori et al. 2006).

The first paper describing the critical role of CNTROB in centriole duplication reported a striking effect of CNTROB depletion in HeLa cells, namely, the accumulation of multinucleate cells resulting from a failure in cytokinesis after delayed and/or prolonged mitoses (Jeffery et al. 2010; Zou 2005). CNTROB was independently identified in a screen for proteins interacting with NEK2 kinase and found to localize to the mitotic spindle, in addition to the centrosomes (the authors refer to CNTROB as NIP2, for NEK2-interacting protein 2) (Jeong et al. 2007). NEK2 phosphorylates CNTROB both in vivo and in vitro, specifically in the N-terminal region of CNTROB (residues 1–193) (Jeong et al. 2007). The phosphorylation of CNTROB by NEK2 impacts its conformation and cellular localization, as CNTROB alone forms large aggregates when overexpressed, whereas co-overexpression of NEK2 leads to microtubule localization of both or the formation of smaller foci (Jeong et al. 2007). This effect is not observed with a kinase-dead NEK2 mutant (Jeong et al. 2007). In other cells where either full-length CNTROB or the C-terminal half (445–903) is overexpressed, perinuclear bundles of nocodazole-resistant-acetylated microtubules are observed, suggesting that CNTROB might have a microtubule-stabilizing function (Jeong et al. 2007).

Consistent with this, mitotic cells depleted of CNTROB form disorganized spindles with either fragmented or unattached centrosomes (Jeffery et al. 2010; Jeong et al. 2007).

The most interesting finding with regard to NEK2 regulation of CNTROB is that overexpression of NEK2 displaces CNTROB from interphase centrosomes, whereas depletion of NEK2 leads to accumulation of higher levels of endogenous CNTROB at centrosomes, implying NEK2 plays a role in regulating CNTROB localization (Jeong et al. 2007). In addition to NEK2 regulatory phosphorylation, CNTROB is also a target of PLK1 in mitosis (Lee et al. 2010). The phosphorylation of CNTROB by PLK1 at multiple residues was demonstrated in vitro, and mutation of four residues abolishes phosphorylation by PLK1 (T3A-S4A, S21A, S22A), whereas no single substitution abolishes this activity (Lee et al. 2010). Perhaps surprisingly, NEK2 phosphorylation of CNTROB is not essential for PLK1 phosphorylation in vivo (Lee et al. 2010).

Phosphorylation of CNTROB appears to influence its effects on microtubules. Purified GST-CNTROB can increase microtubule polymerization rates in vitro (Lee et al. 2010). When purified from cells overexpressing constitutively active PLK1 (but not kinase-dead PLK1), GST-CNTROB shows higher microtubule polymerization activity (Lee et al. 2010). Conversely, a CNTROB construct with alanine substitutions at the PLK1 phosphorylation sites (T3A-S4A-S21A-S22A) shows no sensitivity to the overexpression of constitutively active PLK1 (Lee et al. 2010). Consistent with a PLK1-regulated mitotic spindle function of CNTROB, a PLK1 phosphorylation-resistant siRNA-resistant CNTROB fails to rescue spindle assembly defects following CNTROB depletion in HeLa cells (Lee et al. 2010). In *Drosophila*, it has been found that the homologue of CNTROB may interact with a complex of several PCM proteins (*Drosophila* homologues of CDK5RAP2, TUBG1, γ-TuRC components) and as discussed may be important in select cell types for regulating PCM activity in interphase (Conduit 2013; Januschke et al. 2013). Overall, like CENPJ, CNTROB is a PCM protein required for centriole duplication, but CNTROB is also a NEK2- and PLK1-regulated microtubule-stabilizing protein with a critical role in mitosis.

3.2.4 Microtubule Nucleation by Mitotic PCM

NEDD1 (also called GCP-WD) was identified as the human homologue of *Drosophila* Dgp71WD and is critical for mitotic spindle formation (Haren 2006; Lüders et al. 2006). NEDD1 localizes to interphase and mitotic centrosomes and interacts with GCP2 and TUBG1 by co-IP in human cells (Lüders et al. 2006). When NEDD1 is depleted, centrosomes have reduced TUBG1 but not PCNT, whereas depletion of TUBG1 does not reduce NEDD1 localization to centrosomes (Haren 2006; Lüders et al. 2006). NEDD1 is made up of a WD40 domain required for centrosome localization and a conserved C-terminal domain that mediates γ-TuRC interactions (Haren 2006; Lüders et al. 2006). Consistent with the model that NEDD1 is important for delivery of γ-TuRC components to the centrosomes,

fragments of NEDD1 that lack the WD40 domain disrupt centrosomal TUBG1 recruitment presumably by competitively interacting in the cytoplasm (Lüders et al. 2006). When NEDD1 fragments lacking both the WD40 domains and the conserved CTD are overexpressed, TUBG1 localization is normal (Lüders et al. 2006). The importance of NEDD1 in γ-TuRC organization is further supported by microtubule regrowth experiments, where mitotic cells lacking NEDD1 were deficient in both chromosome- and centrosome-based microtubule nucleation (Haren 2006; Lüders et al. 2006).

NEDD1 is regulated by phosphorylation, and CEP192 interacts with NEDD1 and is important for its phosphorylation and localization in mitosis (Gomez-Ferreria et al. 2012; Zhu et al. 2008). Regulation of NEDD1 was initially shown to involve Cdk1 phosphorylation at S418 (Lüders et al. 2006). S418 is sometimes referred to as S411, reflecting the use of a shorter NEDD1 isoform that lacks the first seven amino acids of the originally reported longer isoform (Lüders et al. 2006). For consistency, the numbering used in this chapter will reflect the residue identities in the longer isoform of NEDD1, not necessarily the numbering in the cited papers. In rescue experiments, NEDD1-S418A mutants could rescue localization of TUBG1 to centrosomes, but not to the mitotic spindle, indicating that this phosphorylation was critical for intra-spindle microtubule nucleation (Lüders et al. 2006). In microtubule regrowth experiments, the NEDD1-S418A does not impact microtubule nucleation at centrosomes or chromosomes, but the mitotic spindles that formed are less dense between the poles (Lüders et al. 2006). In a later study, another group identifies T557 as an amino acid residue where a priming phosphorylation of NEDD1 takes place (Zhang et al. 2009a, b). Although properly localized to centrosomes, a NEDD1 T557A mutant is incapable of binding PLK1, consistent with priming phosphorylation of NEDD1 driving the interaction with PLK1 (Zhang et al. 2009a, b). Using mass spectrometry, three sites are confirmed as phosphorylated by PLK1 in vitro (T389, S404 and S644), and sequence analysis indicates that S433 is a consensus PLK1 binding site (Zhang et al. 2009a, b). NEDD1 constructs with alanine substitutions at either T557 or at all four PLK1 target residues (NEDD1-4A) and fails to co-IP TUBG1 (Zhang et al. 2009a, b). The NEDD1-4A construct is able to localize to centrosomes, but does not recruit TUBG1, and in rescue experiments NEDD1-4A cannot restore TUBG1 localization to the centrosome and spindles in mitotic cells (Zhang et al. 2009a, b). In the same experiment, a NEDD1 construct (NEDD1-4E) designed to mimic PLK1 phosphorylation at all four sites with glutamic acid residues is able to rescue TUBG1 localization (Zhang et al. 2009a, b). Unexpectedly, the NEDD1-4E is not able to rescue the recruitment of TUBG1 to centrosomes when PLK1 and endogenous NEDD1 were both depleted (Zhang et al. 2009a, b). This suggests that while NEDD1 phosphorylation is important for its function and required for its interaction with TUBG1, other targets of PLK1 must also be phosphorylated before TUBG1 can be recruited to centrosomes (Zhang et al. 2009a, b). The phosphorylation of S418 of NEDD1 was later revisited and found to be essential for PLK1 interaction as well, suggesting that both T558 and S418 must be phosphorylated by Cdk1 before PLK1 can interact with NEDD1 (Haren et al. 2009; Zhang et al. 2009a, b).

NEDD1 may also be regulated by a third kinase, NEK9 (Sdelci et al. 2012). NEK9 depletion leads to failure of prometaphase recruitment of both NEDD1 and TUBG1 to centrosomes (Sdelci et al. 2012). FLAG-NEDD1 can co-immunoprecipitate NEK9, and when a conserved serine (S377) is mutated to mimic phosphorylation, the resulting NEDD1 (NEDD1-S377D or NEDD1-S377E) can reverse the phenotype of NEK9 depletion (Sdelci et al. 2012). Conversely, an siRNA-resistant NEDD1-S377A mutant does not localize to centrosomes and cannot rescue the TUBG1 recruitment defects that follow NEDD1 depletion (Sdelci et al. 2012). These results indicate that NEDD1 phosphorylation by NEK9, possibly at S377, is important for its centrosome enrichment during maturation and TUBG1 recruitment during mitosis (Sdelci et al. 2012).

Recently, it was discovered that a ~71 kDa protein called SSX2IP (synovial sarcoma X breakpoint 2 interacting protein) was important for mitotic centrosome function in human cells and *Xenopus* cell-free extracts (Barenz et al. 2013; de Bruijn et al. 2002; Hori et al. 2014). In *Xenopus*, SSX2IP was found to be enriched on mitotic spindles and to be localized to the centrosome by dynein (Barenz et al. 2013). Both human and *Xenopus* SSX2IP co-localize and co-IP PCM1 and TUBG1 (Barenz et al. 2013; Hori et al. 2014). In one study, depletion of human SSX2IP reduced mitotic TUBG1 levels at the spindle poles and led to fragmented PCM and prolonged metaphase (Barenz et al. 2013). In a second study, TUBG1 levels were not strongly reduced, but the density of mitotic spindle microtubules was affected (Hori et al. 2014). The authors explained that their observations were made at 48 h of depletion, whereas the previous study reported 72 h depletion, and they observed a time-dependent loss of TUBG1 with SSX2IP depletion (Barenz et al. 2013; Hori et al. 2014). When microtubules are regrown following washout of nocodazole, centrosomes initially (5 min post-washout) nucleate microtubule asters despite lack of SSX2IP, but by 30 min post-washout, centrosomes have lost their asters, suggesting microtubule-anchoring defects (Hori et al. 2014). Time-dependent loss of microtubule organization has been observed following depletion of PCM1, consistent with PCM1 delivering SSX2IP and other important PCM proteins to the centrosome (Dammermann 2002). The PCM1-binding domain of SSX2IP was mapped to the NTD of the protein, and the TUBG1-binding domain was within a region including the third coiled-coil domain and the CTD of the protein (Hori et al. 2014). SSX2IP fragments lacking the PCM1-binding domain localize to cytoplasmic foci, whereas PACT fusions of this domain partially rescue the effect of SSX2IP depletion on the interphase centrosome's ability to organize microtubule asters and the mitotic spindle microtubule defects observed when SSX2IP is depleted (Hori et al. 2014). Overall, SSX2IP may be important for retention or 'anchoring' of microtubules at centrosomes, rather than centrosome maturation as previously supposed (Barenz et al. 2013; Hori et al. 2014).

PCNT is a major scaffold for the expanded mitotic PCM and is critical for the microtubule nucleation capability of mitotic centrosomes. Pericentrin can directly anchor γ-tubulin ring complexes through multiple domains (Dictenberg et al. 1998; Lin et al. 2014; Takahashi et al. 2002; Zimmerman et al. 2004). PLK1-activated PCNT is important for recruiting CEP192, NEDD1, AURKA and γ -tubulin to

mitotic centrosomes, making PCNT an upstream recruitment factor for proteins required for microtubule nucleation (Joukov et al. 2010, 2014; Lee and Rhee 2011). Like PCNT, AKAP9 co-IPs the γ-TuRC complex proteins GCP2 and GCP3 through its N-terminal region, consistent with a role in anchoring or recruiting γ-TuRCs to the centrosome and spindle (Takahashi et al. 2002). AKAP9 depletion in mitosis does not affect PCNT localization, but results in fragmentation of PCNT and γ-tubulin foci, along with a disorganized spindle (Oshimori et al. 2009). PCNT is also important for CDK5RAP2 recruitment to mitotic centrosomes, independent of phosphorylation by PLK1 (Lee and Rhee 2011). CDK5RAP2 depletion leads to mitotic cells that can have monopolar spindles or otherwise normal spindles where the centrosome is not properly positioned relative to the spindle (Lee and Rhee 2010). Mitotic centrosomes also have reduced γ-tubulin localization and a lack of astral microtubules when CDK5RAP2 is depleted (Fong et al. 2008). PCNT recruitment to mitotic centrosomes is also dependent on CEP192 recruitment, and CEP192 plays a critical role in microtubule nucleation and proper mitotic spindle formation (Gomez-Ferreria et al. 2007; Haren et al. 2009; Zhu et al. 2008). CEP192 is phosphorylated by PLK1, and this phosphorylation creates γ-tubulin-binding sites that are important for mitotic spindle formation (Joukov et al. 2010, 2014; Lee and Rhee 2011). In summary, PCNT, AKAP9, CDK5RAP2 and CEP192 are all critical for the proper formation of mitotic spindles through their roles in microtubule nucleation or anchoring.

3.3 Conclusions and Perspectives

The PCM is a complex network of proteins organized around templates called centrioles to create functional centrosomes that can act as MTOCs and signalling hubs in interphase. Through a complex and carefully regulated series of events called ‘centrosome maturation’, the PCM is further able to provide the microtubule nucleation capabilities required to organize and anchor the mitotic spindle to the centrioles during cell division. The centriole cartwheel structure has long been highly refined. Recently, the understanding of PCM structure has advanced significantly, driven by the technological advances of light microscopy (Leidel and Gönczy 2003). However, many challenges remain. The assembly and dynamics of the PCM are still only partially understood, especially in humans, despite the growing list of recruitment dependencies and interactions (Leidel and Gönczy 2003). There is evidence in *Drosophila* that CNN (CDK5RAP2 homologue) is constantly being actively transported to and from the centrosomes and its incorporation within the PCM is mobile, with CNN recruited first to the centriole then moving outwards from there (Conduit et al. 2010; Conduit et al. 2014a, b; Megraw 2002). It will be of great interest to see how far such a model is applicable and if other components of the PCM undergo migrations beginning at the centriole. Testing the possibility of dynamics in human PCM components will also be of great interest. Further, although PCM maturation is heavily investigated because of the serious mitotic defects and diseases that may arise from them, the process by which

the PCM returns to interphase levels is not well understood. It is likely to involve ubiquitinylation followed by proteasomal degradation, as has been clearly shown to be the case for regulation of centriole duplication factors, such as Sas-6 and CENPJ (Korzeniewski et al. 2010; Puklowski et al. 2011). Finally, although we have recently expanded our understanding of PCM and its subdomains substantially, there is certainly room for continued study of how other proteins are organized within the PCM. Notably, the orientation of AKAP9 within the PCM is not studied, but given its large size and similarities to PCNT, it will be an interesting protein to investigate. Additionally, in vitro reconstitution of PCM sub-complexes from recombinant proteins might be a promising strategy to gain insight into organization of PCM subdomains and the kinetics of their assembly (Leidel and Gönczy 2003). There are many proteins in the PCM, and accordingly many opportunities remain for useful insights into PCM structure and organization using ever-improving super-resolution microscopy methods. Hopefully, one day these techniques will converge with cryo-electron tomography and x-ray crystallography to provide a detailed 3D atlas of the PCM at the molecular level.

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Principles of Microtubule Organization: Insight from the Study of Neurons

4

Carlos Sánchez-Huertas, Francisco Freixo, and Jens Lüders

Abstract

A multitude of protein activities contribute to the organization of cell type and cell cycle-specific microtubule arrays. One key factor is the γ-tubulin ring complex (γTuRC), a microtubule nucleator that determines where and when new microtubules are generated. Other proteins interact with newly formed or existing microtubules to promote microtubule stabilization, destabilization, severing, bundling, or transport. Together these activities allow arrangement of microtubules into arrays with specific distribution, polarity, and dynamic properties. Importantly, microtubule arrays are not static and can undergo extensive remodeling. During neural development, for example, self-renewing and neurogenic divisions of neural progenitors require specific spindle positioning, which is determined by centrosome-based microtubule organization. In newly born neurons, the centrosomal microtubule array mediates the migration process. However, during neuron maturation the centrosome-centered microtubule network is converted into non-centrosomal, highly bundled arrays, which are crucial for long-range transport within the extensive dendritic and axonal compartments. Accordingly, neuronal development, homeostasis and function are particularly sensitive to genetic and other insults of the microtubule cytoskeleton. In this chapter we will highlight, using neurons as an example, different microtubule-organizing activities, in particular microtubule nucleation and its spatiotemporal regulation, and discuss how defects in the microtubule network are implicated in neurodevelopmental disorders and neurodegenerative diseases.

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4.1 Cellular Tools for Organizing Microtubules

4.1.1 Microtubule Nucleation

A key mechanism underlying assembly, maintenance, and remodeling of ordered microtubule arrays is microtubule nucleation. Spatiotemporal regulation of this process allows cells to control when, where, and in what orientation new microtubules are formed (Luders and Stearns 2007; Teixidó-Travesa et al. 2012; Yau et al. 2014). Above a critical tubulin concentration, polymerization from purified tubulin occurs spontaneously in vitro. In cells, however, the tubulin concentration is too low to allow spontaneous polymerization and formation of microtubules requires a nucleator. In animal cells microtubules are nucleated by the multi-subunit γ-tubulin ring complex (γTuRC). γTuRC is composed of γ-tubulin, a member of the tubulin superfamily that is not incorporated into the microtubule polymer, and additional subunits known as gamma complex proteins 2, 3, 4, 5, and 6 (GCP2-6) (Fig. 4.1) (Baas and Joshi 1992; Kollman et al. 2011; Teixidó-Travesa et al. 2012). GCP2-6 are related to each other and form a protein family. Based on the crystal structure of GCP4 and the sequence similarity between GCP4 and other GCPs, all members of this protein family were predicted to have the same elongated shape (Guillet et al. 2011; Kollman et al. 2011). According to the current model, the lock washer-like structure of the γTuRC is formed by oligomerization of the GCPs through lateral association of their N-terminal domains and binding of γ-tubulin to their C-terminal domains (Kollman et al. 2011). Whereas the exact positions and stoichiometries of distinct GCP subunits are still unknown, γTuRC contains ~13 γ-tubulin molecules, the arrangement of which matches the symmetry of a microtubule in cross section. Based on this observation, a template-based nucleation model was proposed: by mimicking the end of a microtubule, γTuRC provides a platform for the assembly of heterodimers of α- and β-tubulin, which initiates microtubule polymerization (Fig. 4.1) (Moritz et al. 2000; Kollman et al. 2010; Kollman et al. 2011).

Recent work has suggested that efficient nucleation requires additional factors that cooperate with γTuRC by stabilizing early nascent microtubules (Goodwin and Vale 2010; Tanaka et al. 2012; Wieczorek et al. 2015). While the γTuRC template mimics the microtubule symmetry in cross section, its blunt structure differs from an actively growing plus end, which is splayed and outwardly curved. Polymerization-promoting factors such as the microtubule-binding protein TPX2 may help to transform nascent microtubules into a complete microtubule plus end, which will facilitate further polymerization and allow robust microtubule growth (Yau et al. 2014; Wieczorek et al. 2015).

Spatiotemporal control over the formation of new microtubules is crucial to microtubule organization. Thus an important question is how γTuRC is regulated. Regulatory activities have been assigned to various γTuRC-associated proteins such as GCP-WD/NEDD1, CDK5RAP2, Mozart1, GCP8/Mozart2, and NME7. These proteins are typically not required for γTuRC assembly but target γTuRC to specific nucleation sites (GCP-WD, Mozart1, CDK5RAP2) or activate its

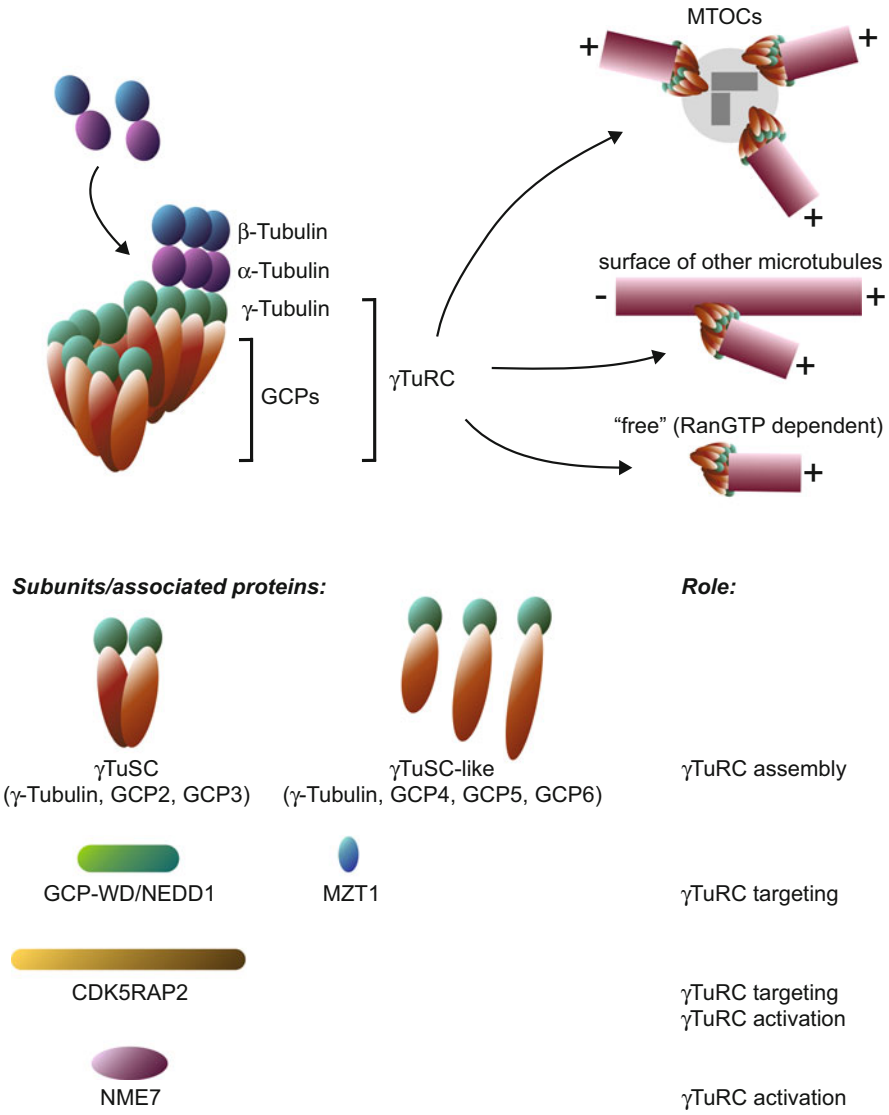

Fig. 4.1 Composition and function of the γ-tubulin ring complex (γTuRC). The γTuRC, the main microtubule nucleator, initiates microtubule polymerization by a template mechanism. It is composed of ~13 γ-tubulin molecules that are arranged into a helical, lock washer-like structure with the help of multiple GCP subunits (GCP2, GCP3, GCP4, GCP5, and GCP6). The surface formed by the γ-tubulin molecules in the γTuRC serves as assembly platform for α-β-tubulin heterodimers. γTuRC is regulated by specific targeting and activation factors to nucleate microtubules from centrosomes, the surface of other microtubules, and "free" within the cytosol (e.g., stimulated by RanGTP during mitosis). The roles of some γTuRC subunits and associated proteins are depicted: γ-tubulin and GCPs 2-6, by forming γTuSC- and γTuSC-like sub-complexes, have roles in γTuRC assembly, GCP-WD/NEDD1 and MZT1 in targeting, CDK5RAP2 in targeting and activation, and NME7 in activation

nucleation activity (CDK5RAP2, NME7) (Fig. 4.1) (Sherwood et al. 2004; Trotta et al. 2004; Yang et al. 2005; Yu et al. 2005; Lüders et al. 2006; Haren et al. 2006; Solowska et al. 2008; Lee et al. 2009; Hutchins et al. 2010; Butler et al. 2010; Choi et al. 2010; Teixidó-Travesa et al. 2012; Liu et al. 2014a). The precise function of GCP8 is still unclear: unlike other γTuRC subunits, it is not required for mitotic spindle assembly suggesting that it may have a role in non-mitotic cells (Teixidó-Travesa et al. 2010).

A well-characterized γTuRC-targeting factor in human cells is NEDD1 (also known as GCP-WD), which targets γTuRC to the centrosome (Lüders et al. 2006; Haren et al. 2006). NEDD1 binds γTuRC through its C-terminus and attaches it at the centrosome via its N-terminal domain. In addition, NEDD1 is also involved in targeting γTuRC to spindle microtubules in mitosis (see below) (Lüders et al. 2006). Targeting to these sites involves regulation by differential phosphorylation of NEDD1 (Lüders et al. 2006; Zhang et al. 2009; Johmura et al. 2011; Gomez-Ferreria et al. 2012; Sdelci et al. 2012; Pinyol et al. 2012). Curiously, NEDD1 is not essential for targeting γTuRC to centrosomes in *Drosophila* (Vérollet et al. 2006) and NEDD1 is not present in fungi indicating that targeting of γTuRC can also be mediated by other factors. Indeed, various adapter proteins that are unrelated to NEDD1 have been described in different organisms. Recent work demonstrated that with the exception of NEDD1, all of these proteins contain short sequence motifs that are conserved throughout the animal, fungi, and plant kingdoms and that mediate γ-tubulin complex binding and in some cases also activation (Choi et al. 2010; Lin et al. 2014, 2015). In human cells the proteins CDK5RAP2 and myomegalin, which have been implicated in γTuRC recruitment to centrosomes and Golgi, respectively, contain the so-called CM1 motif. The centrosomal scaffold protein pericentrin contains two motifs, CM1 and SPM, and has also been implicated in centrosome recruitment of γTuRC (Sawin et al. 2004; Samejima et al. 2008; Choi et al. 2010; Lin et al. 2014, 2015). It is currently unclear whether multiple adaptors interact with γTuRC simultaneously or whether the different γTuRC recruitment factors function independently of each other, potentially to recruit γTuRCs with distinct composition and/or functions at the respective nucleation site.

Another important unresolved question is how γTuRC nucleation activity is regulated. This process is best understood for the budding yeast γ-tubulin complex. Budding yeast lacks orthologs of GCP4, GCP5, and GCP6 and does not assemble γTuRCs. Instead it contains a heterotetrameric γ-tubulin small complex (γTuSC) assembled from two molecules of γ-tubulin and one each of GCP2 and GCP3 (Fig. 4.1) (Knop and Schiebel 1997; Vinh et al. 2002). γTuSC is a poor nucleator and requires interaction with the adaptor protein Spc110 to oligomerize and form nucleation-competent γTuRC-like rings (Kollman et al. 2010; Lin et al. 2014). However, oligomerization alone does not strongly enhance nucleation activity. This can be explained by a structural mismatch that was observed between the oligomeric γTuSC ring structure and the microtubule end: the spacing between γ-tubulin molecules in the γTuSC ring does not match the narrower spacing of α-tubulin molecules at the microtubule end. Indeed, a conformational switch that adjusts the

positioning of γ-tubulin molecules in the γTuSC ring can be induced artificially in vitro and yields a more active nucleator (Kollman et al. 2015). More recently, it was shown that γTuSC oligomerization and activation involve interaction of Spc110 with the N-terminus of GCP3 and require the CM1 and SPM motifs of Spc110 as well as cell cycle-dependent phosphorylation by the yeast kinases Cdk1 and Mps1 in a region between these two motifs (Lin et al. 2014).

In contrast to budding yeast cells, human cells contain pre-assembled, ring-shaped γ-tubulin complexes in the form of γTuRC (Murphy et al. 2001). However, most of cytosolic γTuRC seems to be in a relatively inactive state, suggesting that γTuRC assembly and activation are two separate steps. Indeed, CDK5RAP2 or a fragment containing only the CM1 motif were shown to strongly stimulate γTuRC nucleation activity both in vitro and in vivo (Choi et al. 2010). In addition, the nucleoside diphosphate kinase and γTuRC interactor NME7 also moderately activated γTuRC-dependent nucleation (Liu et al. 2014a). The molecular basis of γTuRC activation is currently unknown. Converting γTuRC into an active nucleator may involve a conformational switch, similar to what has been described for oligomeric γTuSC (Kollman et al. 2015). However, testing this model will require structural analysis of γTuRC at a resolution that is higher than the ones currently available.

4.1.2 Microtubule Organizing Centers

Spatial control over microtubule assembly is achieved with the help of specific cellular structures that function as microtubule-organizing centers (MTOCs) by nucleating and anchoring microtubules (Luders and Stearns 2007). The main MTOC in animal cells, the centrosome, is a small spherical structure formed by a central pair of centrioles surrounded by a proteinaceous matrix that nucleates microtubules. For detailed information on the structure and function of this "pericentriolar material" (PCM), we refer to the Chap. 3 by Comartin and Pelletier.

A newly born cell in G1 phase contains a single centrosome, which is duplicated precisely once during the cell cycle (Firat-Karalar and Stearns 2014; Fu et al. 2015). At mitotic entry the cell contains two centrosomes that will be used to organize the two spindle poles of the bipolar mitotic spindle. Even though centrosomes are not strictly required for mitotic spindle assembly, centrosomal microtubule organization enhances the efficiency of this process and promotes spindle bipolarity and the fidelity of chromosome segregation. Moreover centrosomal microtubules are important for controlling spindle positioning (Vitre and Cleveland 2012; Sir et al. 2013). For this reason cells tightly control centrosome maintenance and copy number (Firat-Karalar and Stearns 2014; Fu et al. 2015). For a detailed discussion of mitotic spindle assembly, we refer to the Chap. 1 by Meunier and Vernos. Regarding the consequences of numerical centrosome aberrations, please see the Chap. 5 by Gambarotto and Basto.

After nucleation the minus end of microtubules is typically anchored at MTOCs, whereas the plus end extends away from the MTOC into the cytoplasm (Lüders and

Stearns 2007). Since nondividing cells typically contain only a single centrosome, this results in the organization of a radial microtubule array. Even in proliferating cells, in which centrosome duplication occurs, the radial organization is maintained because the duplicated centrosomes are physically linked and remain in close proximity to each other. Only at the G2/M phase transition, centrosomes separate (centrosome disjunction) and help converting the radial array into a bipolar mitotic spindle (Firat-Karalar and Stearns 2014; Fu et al. 2015).

Apart from centrosomes several non-centrosomal MTOCs have been described (Lüders and Stearns 2007). It appears that a centrosome-centered, radial microtubule network is mainly found in fibroblast-like cells and that alternative microtubule configurations organized by non-centrosomal MTOCs are common in other cell types. According to the concept of MTOC plasticity, originally introduced by Mazia (Mazia 1984), specific microtubule-organizing proteins including the nucleator γTuRC are present not only at centrosomes but can also associate with various other cellular structures to form MTOCs of variable size, shape, and distribution (Lüders and Stearns 2007). Examples are the cytosolic surfaces of the Golgi network (Chabin-Brion et al. 2001; Rios et al. 2004; Oddoux et al. 2013; Zhu and Kaverina 2013; Rios 2014) and of the nuclear envelope (Tassin et al. 1985; Musa et al. 2003; Bugnard et al. 2005). More details on non-centrosomal microtubule organization in different cell types can be found in the Chap. 2 by Dyachuk, Bierkamp and Merdes.

Recently an additional, unusual "MTOC" was added to the list: the lateral surface of existing microtubules can function as a recruitment site for γTuRC, to promote nucleation of "daughter" microtubules in the form of lateral branches that have the same polarity as the "mother" microtubule (Goshima et al. 2008; Petry et al. 2013). This nucleation mode was initially described in the cortical microtubule array of certain plant cells and later also in fission yeast and in the mitotic and meiotic spindles of animal cells (Janson et al. 2005; Murata et al. 2005; Sánchez-Huertas and Lüders 2015). This type of nucleation serves two functions: first, it rapidly increases the amount of microtubules during the early stages of microtubule array assembly, and second, it reinforces and maintains existing microtubule arrangements including their polarity.

4.1.3 Activities That Modify the Properties and Behavior of Microtubules

A large number of proteins associate with microtubules to regulate their properties and behavior. This includes proteins that bind specifically to minus ends, proteins that bind to the microtubule lattice, and proteins that specifically associate with microtubule plus ends.

Apart from the nucleator γTuRC, only a few other proteins interact specifically with microtubule minus ends (Akhmanova and Hoogenraad 2015; Akhmanova and Steinmetz 2015). These proteins belong to the CAMSAP family that comprises three members CAMSAP1, CAMSAP2, and CAMSAP3. For CAMSAP2 it was

recently shown that it stabilizes the minus ends of newly formed microtubules in the dendrites of neurons, subsequent to nucleation by the γTuRC (Jiang et al. 2014; Yau et al. 2014). In the absence of CAMSAP2, dendrites contained fewer microtubules and microtubule-dependent extension and branching were impaired.

Proteins that bind to the microtubule lattice frequently help in stabilizing microtubules. TPX2, for example, can prevent depolymerization and thus functions as anti-catastrophe factor. This activity was recently suggested to assist in γTuRC-dependent nucleation by stabilizing nascent plus ends in the very early phases of nucleation, before robust microtubule elongation can occur (Wieczorek et al. 2015). Other examples for lattice binders are MAP2 and TAU, proteins that stabilize neuronal microtubules in dendrites and axons, respectively (Dehmelt and Halpain 2005).

Other lattice-binding proteins have ATP-dependent motor activity and use microtubules as tracks to transport cargo. The dynein motor moves cargo toward minus ends, whereas most of the kinesin motors are plus end-directed. Some lattice-binding proteins including some motors can interact with different microtubules simultaneously via two independent lattice-binding regions. In this way they can cross-link microtubules and thus contribute to microtubule bundling and sliding (Sharp et al. 1999; Mountain et al. 1999).

Some lattice-binding proteins use energy derived from ATP hydrolysis to sever microtubules and generate multiple shorter microtubule fragments. In humans three such proteins are known: katanin, spastin, and fidgetin (Roll-Mecak and Mcnally 2010; Sharp and Ross 2012).

Another group of enzymes interacts with the microtubule lattice to posttranslationally modify the tubulin subunits. These modifications are believed to change the properties of microtubules to regulate, for example, interactions with microtubule-associated proteins (MAPs) or motor proteins (Janke and Bulinski 2011; Janke 2014; Song and Brady 2015).

In addition, there is a very large group of proteins that interact specifically with the plus ends of microtubules (Gouveia and Akhmanova 2010; Jiang and Akhmanova 2011; Kumar and Wittmann 2012; Akhmanova and Steinmetz 2015). These so-called + TIPs have diverse functions; some prevent microtubule depolymerization or even actively promote polymerization by acting as tubulin polymerases. An example is the *Xenopus laevis* protein XMAP215 (CKAP5 in humans), which was proposed to assist, similar to TPX2, γTuRC in nucleating microtubules (see above) (Wieczorek et al. 2015). Some + TIPs function as depolymerases by destabilizing the growing plus end. Through this function the depolymerase MCAK, for example, was proposed to negatively regulate nucleation by γTuRC, opposite to the action of TPX2 or XMAP215 described above (Wieczorek et al. 2015). Other + TIPs regulate the dynamics of the plus end by altering the rates at which microtubules grow or shrink. Some of these proteins also mediate the interaction of growing microtubule plus ends with other cellular structures. In this way the dynamic microtubule plus ends can provide regulatory activity (Gouveia and Akhmanova 2010; Jiang and Akhmanova 2011; Akhmanova and Steinmetz 2015).

4.2 The Microtubule Network in Neurons

Proper organization of the microtubule network is particularly important in neurons as indicated by the following observations. (1) Microtubules drive neuronal morphogenesis during normal development as well as during regeneration after injury. For example, microtubule-stabilizing drugs can promote axon regeneration in spinal cord neurons (Hellal et al. 2011; Ruschel et al. 2015). (2) Microtubules are important for neuron homeostasis as indicated by severe peripheral neuropathies in cancer patients treated with chemotherapeutic microtubule poisons (Schmidt and Bastians 2007; Baas and Ahmad 2013; Funahashi et al. 2014). (3) Several neurodegenerative disorders are caused by gene mutations that impair microtubule-based transport (Perlson et al. 2010; Kuijpers and Hoogenraad 2011). (4) Transport defects associated with the abnormal accumulation of proteins and organelles in axons have been suggested to contribute to the pathology of neurodegenerative disorders such as Huntington's, Parkinson's, and Alzheimer's disease (Perlson et al. 2010; Kuijpers and Hoogenraad 2011).

Considering the importance of the microtubule cytoskeleton in neurons, we will present in the following section our current understanding of how these cells organize their microtubule network.

4.2.1 Organization of Neuronal Microtubule Arrays

When a progenitor cell differentiates, it remodels its microtubule array to allow new cellular functions. Major reorganization of microtubules occurs during the differentiation of neurons, due to their extreme polarization and subcellular compartmentalization. This polarized structure results from the formation of one long axon and multiple shorter dendrites. These two types of subcellular compartments differ in their morphology, internal organization, and function. The distinct features of axons and dendrites are, to a large extent, determined by the differential organization of the microtubule cytoskeleton (Fig. 4.2) (Baas et al. 1988; Burton 1988; Poulain and Sobel 2010; Kuijpers and Hoogenraad 2011).

Even though centrosomes are present in postmitotic neurons, they are not at the center of the microtubule network. Instead neurons display non-centrosomal microtubules arrays that are characterized by a high degree of bundling. Whereas microtubule bundling is prominent in both axons and dendrites, the configuration of microtubules within these bundles differs between the two neuronal compartments. In vertebrate neurons the axonal microtubules have uniform polarity, with their plus ends facing the axons tip, whereas the dendritic microtubules are of mixed polarity. However, the distal dendritic tips contain unipolar microtubules oriented in the same way as in axons (Conde and Cáceres 2009; Sakakibara et al. 2013). By determining the directionality of motor-dependent cargo transport, the compartment-specific orientation of microtubules establishes and maintains neuronal polarization and compartment identity and thus is at the heart of neuronal function.

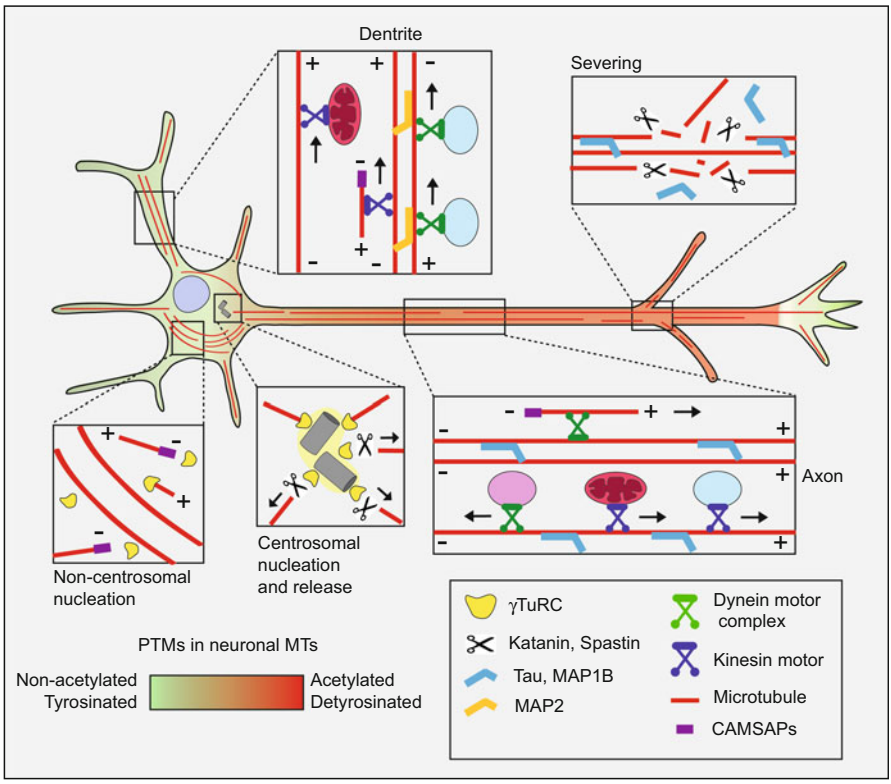

Fig. 4.2 Organization and regulation of microtubule arrays in neurons. New microtubules in neurons can be generated by γTuRC-mediated nucleation at the centrosome, followed by cleavage through severing enzymes and release of the newly formed microtubules (*box "Centrosomal nucleation and release"*). In the cytoplasm, γTuRC can also promote non-centrosomal nucleation of microtubules, which are then stabilized at their minus ends by CAMSAPs (*box "Non-centrosomal nucleation"*). In the axon microtubules display a uniform polarity with plus ends distal to the soma (*box "Axon"*). Short microtubules either formed locally by severing (*box "Severing"*) or derived from the soma are transported by dynein-dependent sliding (*box "Axon"*). Other cargoes such as mitochondria and vesicles are also transported throughout the axon by dynein or kinesins in an anterograde or retrograde fashion, respectively (*box "Axon"*). The transport specificity of axonal cargo is achieved among other factors by posttranslational modification (*PTM*) of microtubules. Axonal microtubules display a high degree of acetylation and are enriched in the MAP tau. In the somato-dendritic compartment, microtubules are less acetylated and more tyrosinated than in the axon and are decorated with MAP2 (*box "Dendrite"*). In dendrites, microtubule organization also differs from the axon, as microtubules have mixed polarity. Minus-end-distal microtubules are transported into dendrites by kinesins. However, at the dendrite tip microtubule polarity is more uniform with plus ends distal, similar to the axon. As expected, these features also regulate the transport of specific dendritic cargo, either by dynein or kinesins (*box "Dendrite"*)

4.2.2 MTOCs in Neurons: Centrosomal vs. Non-centrosomal

In young neurons the centrosome organizes the microtubule array required for neuronal migration (please see the Chap. 6 by Theisen and Straube for more information), and in some types of neurons, centrosome position determines the site of axon specification, even though there is still controversy on this matter (Zmuda and Rivas 1998; de Anda et al. 2005; Gärtner et al. 2012). Despite these observations, the role of the centrosome in generating the microtubules present in the distinct cellular compartments of more mature neurons remains unclear. Early work suggested that the centrosome is the main source of neuronal microtubules. It was proposed that microtubules are nucleated at the centrosome from where they are released by severing and, with the help of motor proteins, actively transported along microtubule tracks to other locations in the cell (Yu et al. 1993; Baas and Yu 1996; Karabay et al. 2004; Zheng et al. 2008; Lin et al. 2012). However, more recent studies have challenged this view. The presence of an adult, morphologically normal nervous system in flies without centrosomes suggests that centrosomes are not essential for neuron morphogenesis (Basto et al. 2006). This is also supported by the observations that elimination of the centrosome does not impair axon extension in cultured rat hippocampal neurons (Stiess et al. 2010) and does not alter microtubule polarity in axons and dendrites of *Drosophila* larvae neurons (Nguyen et al. 2011). In summary, centrosome-derived microtubules play an important role in immature neurons by contributing to neuronal motility and may also contribute to the extension of the first neurites. In subsequent more mature stages, however, it is very likely that non-centrosomal mechanisms become instrumental for generating and maintaining neuronal microtubule arrays.

4.2.3 Non-centrosomal Nucleation

Consistent with the existence of non-centrosomal nucleation, γ-tubulin at centrosomes progressively decreases during neuronal maturation (Leask et al. 1997; Stiess et al. 2010; Yau et al. 2014). Moreover, whereas in young neurons microtubules emanate from the centrosomes, in more mature neurons most of the microtubules are not connected to the centrosome (Stiess et al. 2010). In *Drosophila* neurons it was proposed that non-centrosomal γTuRC nucleates microtubules from the surface of dendritic Golgi outposts to promote dendritic arborization, but this observation has recently been questioned (Ori-McKenney et al. 2012; Nguyen et al. 2014; Quassollo et al. 2015). More recently it was shown that γ-tubulin can nucleate acentrosomal microtubules in the cytoplasm of the somato-dendritic compartment of young and mature hippocampal neurons (Yau et al. 2014), but a specific MTOC was not identified.

γ-Tubulin complexes are known to nucleate microtubules from non-centrosomal sites in various organisms and cell types. For example, in higher plant cells, which do not have centrosomes, microtubules are nucleated from the nuclear envelope and from the lateral surface of other microtubules (Fishel and Dixit 2013; Hashimoto

2013). In dividing animal and plant cells, nucleation from the lattice of other microtubules is mediated by augmin, a multi-subunit protein complex that recruits γTuRC to nucleate branches that grow almost parallel to and with the same polarity as the "mother" microtubule. This augmin-dependent intra-spindle nucleation pathway is crucial for proper mitotic and meiotic spindle assembly and function (Goshima et al. 2008; Colombié et al. 2013; Petry et al. 2013). Interestingly, augmin-dependent branching nucleation was recently found to also drive the assembly of the cortical microtubule array of interphase plant cells (Liu et al. 2014b), suggesting that augmin is not a mitosis/meiosis-specific factor. Thus it is tempting to speculate that augmin may also have a role in postmitotic neurons, in the generation and maintenance of microtubule bundles in axons and dendrites. Indeed, this view is supported by unpublished data from our group.

An important question is how the minus ends of microtubules that are not associated with any MTOC are stabilized. In principle, this function could be carried out by minus end-associated γTuRC. While γ-tubulin is present in the axonal fractions of rat hippocampal cultures (Stiess et al. 2010) and was also detected in dendrites (Yau et al. 2014), it does not seem to be stably bound to the minus ends of non-centrosomal MTs in any of these compartments (Baas and Joshi 1992). Apart from γTuRC, ninein, a minus end-associated protein, was shown to be expressed in neurons and stabilize axonal microtubules (Baird et al. 2004; Srivatsa et al. 2015). More recently, members of the patronin/CAMSAP protein family have been characterized as important minus end-associated factors that stabilize non-centrosomal microtubules (Goodwin and Vale 2010; Tanaka et al. 2012). CAMSAP2 was shown to stabilize minus ends of non-centrosomal MTs nucleated by γTuRC, promoting axon specification and dendrite morphogenesis (Jiang et al. 2014; Yau et al. 2014).

4.2.4 Microtubule Severing

Apart from nucleation, new microtubules can also be generated by breakage of preexisting microtubules through the action of severing enzymes. Severing of microtubules occurs in cycling cells as well as in postmitotic cells, in an ATP-dependent enzymatic process. Initially considered a mechanism for destruction or recycling of parts of the microtubule lattice, microtubule severing was later found to underlie constructive processes, including seeding of new microtubule growth and release of microtubules from their nucleation sites for their subsequent transport. Three classes of microtubule-severing enzymes have been described – katanin, spastin, and fidgetin. All three are expressed in the nervous system in several organisms, and katanin and spastin were shown to have a role in neuronal microtubule organization (Ahmad et al. 1999; Sherwood et al. 2004; Trotta et al. 2004; Yang et al. 2005; Yu et al. 2005; Solowska et al. 2008; Lee et al. 2009; Butler et al. 2010).

Katanin is a heterodimeric enzyme, with a p60 catalytic subunit and a p80 regulatory-and-targeting subunit. Inhibition of katanin p60 subunit in cultured rat

neurons was found to inhibit axon growth, while increasing the number of centrosome-associated microtubules and the overall microtubule length in the soma and the axon (Ahmad et al. 1999). This seems to indicate that katanin is required to cleave centrosome-nucleated microtubules to allow their release and transport to other parts of the cell. In the axon katanin may sever long microtubules to generate multiple short microtubule fragments, which can be transported to the axon tip and, by polymerization, promote axon growth (Karabay et al. 2004). In dendrites, katanin seems to be required for the establishment of correct morphology and arborization. *Drosophila* loss-of-function mutants of katanin p60 showed reduced neurotransmitter efficiency at neuromuscular junctions with an increased elaboration of dendrites (Mao et al. 2014). In contrast, *Drosophila* katanin p60-like 1 (*kat-60 L1*) mutants have decreased dendrite branch number and length in larval class IV sensory neurons (Stewart et al. 2012), and mutations in the *kat-60 L1* gene inhibited dendritic pruning in *Drosophila* larval neurons, a process that removes dendritic branches to allow rewiring of the nervous system during metamorphosis (Lee et al. 2009). These differential effects of severing enzymes on dendritic growth and arborization may be due to differences in their specific localization or regulation.

To avoid an excessive severing of microtubules, this activity needs to be controlled. In axons, the axon-specific protein tau protects microtubules against severing, by limiting access of katanin to microtubules (Qiang et al. 2006). Phosphorylation of tau releases this MAP from microtubules and has been proposed to regulate katanin-mediated severing (Qiang et al. 2010). Microtubule acetylation also regulates severing, as acetylated microtubules are more sensitive to katanin activity (Sudo and Baas 2010; Mao et al. 2014). Furthermore, it was shown that the adenomatous polyposis coli (APC) protein controls the stability and activity of katanin p60 in interneurons to ensure a rapid remodeling of neurites, necessary for interneuron migration (Eom et al. 2014).

Together with katanin the enzyme spastin also regulates axon growth and morphology. In cultured rat neurons spastin was shown to localize at the nascent sites of axonal lateral branches, where it seems to cleave long microtubules, giving rise to short microtubules that can be transported into these new branches (Yu et al. 1994; Yu et al. 2008). This role seems to be conserved in other organisms, such as zebrafish (Butler et al. 2010) and *Drosophila*. While behavior of axonal microtubules seems to be normal in flies heterozygous for a spastin null-allele, these neurons showed severe impairment of regenerative axon growth post-axotomy (Stone et al. 2012). The cooperation between the activities of spastin and katanin is also found in dendrites. Dendritic branching was reduced in null mutants of the spastin gene in *Drosophila*, which goes in line with the hypothesis that spastin generates short microtubules at the branching points of dendrites, to seed the formation of the new microtubules required for branching (Jinushi-Nakao et al. 2007). Tubulin polyglutamylation renders microtubules more sensitive to severing by spastin (Qiang et al. 2006; Lacroix et al. 2010).

4.2.5 Microtubule Transport

Short microtubules generated by nucleation or severing can subsequently be distributed by motor proteins (Fig. 4.2). The specific localizations and activities of various motors promote the compartment-specific organization of neuronal microtubules. For example, microtubules can be transported with the plus end leading into the nascent axon and dendrites to support neurite growth, and transport of minus end-distal microtubules selectively into dendrites was proposed to contribute to dendritic identity (Baas 1998). For quite some time, the existence of microtubule transport was uncertain; the first photobleaching experiments, performed on short axon segments (a few micrometers long) of neurons that were microinjected with fluorescent tubulin, showed no movement of fluorescent particles through the bleached zone. This leads to the hypothesis that either microtubule transport in neurons was a very slow process or that axon microtubules were completely stationary (Hirokawa et al. 1997). However, in subsequent experiments, by photobleaching a longer segment of the axon and extending the imaging period, rapidly moving fluorescent tubulin (presumably in the form of very short microtubules with a length of 7–10 μm) was visualized. The movement was intermittent, asynchronous, and bidirectional and occurred at a rate that was consistent with motor-dependent transport (Wang and Brown 2002; Hasaka et al. 2004; He et al. 2005; Myers and Baas 2007; Qiang et al. 2010; Liu et al. 2010).

In axons, anterograde movement of microtubules is about twice as frequent as retrograde movement (Wang and Brown 2002). Even though the polarity of such microtubules is difficult to assess (only actively growing microtubule plus ends can be visualized; the currently available markers such as members of the EB family only bind to growing plus ends), it was proposed that selective transport of microtubules with specific polarity may underlie the uniform plus end-out orientation of axonal microtubules (Baas and Mozgova 2012). This model assumes that the anterograde-moving microtubules would be plus end distal, whereas the retrograde-moving microtubules would have the opposite polarity (minus end distal). One of the premises of this model is that the retrograde microtubule transport is a clearing mechanism that maintains the uniform polarity of microtubules in the axon, by removing incorrectly oriented, minus end-distal short microtubules. Such microtubules may arise by severing of longer microtubules and flipping of the resulting very short microtubule fragments in wider areas of the axon (Baas and Mozgova 2012). In addition, local nucleation, if not precisely controlled, may also generate microtubules with incorrect polarity.

Concerning the motors that drive this transport, cytoplasmic dynein was one of the first to be tested. The two heavy chains of the multi-subunit dynein complex hydrolyze ATP and mediate movement along microtubules, whereas interaction with cargo such as vesicles and organelles is mediated by the cargo domain. However, since the cargo domain can also bind to microtubules and actin filaments, dynein can also operate in a sliding mode: while the cargo domain is bound to a longer immobile microtubule or actin filament, the motor domain binds to short microtubules and, by moving toward their minus end, “slides” these along the

immobile structure with their plus ends leading (Vale et al. 1992; Keays et al. 2007; Poirier et al. 2007). When dynein heavy chain was partially depleted in neurons, the anterograde movement of microtubules decreased, without affecting the retrograde movement (He et al. 2005; Bahi-Buisson et al. 2014). This would be consistent with a role of dynein in microtubule sliding toward the axon tip. Since it was demonstrated that this dynein-dependent anterograde sliding can occur against both actin filaments and long microtubules but retrograde movement only against microtubules, it was hypothesized that dynein may only be used for sliding against actin filaments (Hasaka et al. 2004; Baas and Mozgova 2012). On the other hand, in a separate study inhibition of dynein generated an increase in misoriented microtubules in the axon in *Drosophila* neurons, suggesting that dynein may also have a role in the retrograde transport mechanism, which would allow clearing of microtubules with incorrect, minus end-distal polarity. Apart from dynein, kinesin motors may also be involved in microtubule transport. However, kinesin motors move (with a few exceptions) toward microtubule plus ends and therefore short microtubules would have to be transported as cargo rather than by sliding. Since this type of transport would occur regardless of short microtubule polarity, it would have the risk of introducing minus end-distal microtubules into the axon. Indeed, the depletion of some of the so-called "mitotic" kinesins that are also expressed in neurons did not decrease the rate of short microtubule transport in the axon, but rather increased it. Eg5/kinesin-5/KIF11, for example, is required for the separation of centrosomes during prophase and formation of the bipolar mitotic spindle (Bertran et al. 2011). In postmitotic neurons, depletion of Eg5 increased bidirectional traffic of short microtubules in the axon, without affecting the movement of vesicles or mitochondria, suggesting that one of its functions is to act as a brake for short microtubule transport (Myers and Baas 2007). Inhibition of Eg5 with drugs also leads to an increase in axon growth in rat peripheral neurons (Haque et al. 2004; Myers and Baas 2007; Tischfield et al. 2010) and to an increase in axon length in immature rat cortical neurons, but not in later stages of neuron maturation in vitro (Yoon et al. 2005). In agreement with these observations, Eg5 was also shown to control the distribution of microtubules in the axonal growth cone (Nadar et al. 2012) and its overexpression in rat peripheral neurons leads to a decrease in axon length and anterograde microtubule transport (Myers and Baas 2007).

Kinesin-12/KLP/KIF15 is also expressed in neurons. Knockdown of kinesin-12 led to an increase in anterograde and retrograde axonal microtubule transport and to faster axon growth, a phenotype shared with Eg5-depleted neurons. However, in contrast to Eg5 depletion, depletion of kinesin-12 did not increase axonal branching or growth cone size (Abdollahi et al. 2009; Liu et al. 2010).

In dendrites, as mentioned before, microtubule polarity is mixed, and therefore the mechanisms driving microtubule transport in this compartment are likely to be different from those in the axon (Fig. 4.2). It is known that microtubules in newly forming dendrites are mostly plus end-distal and that minus end-distal microtubules occur gradually during dendrite growth, to establish the characteristic mixed polarity configuration (Sharp et al. 1995; Jaglin and Chelly 2009; Tischfield et al. 2011).

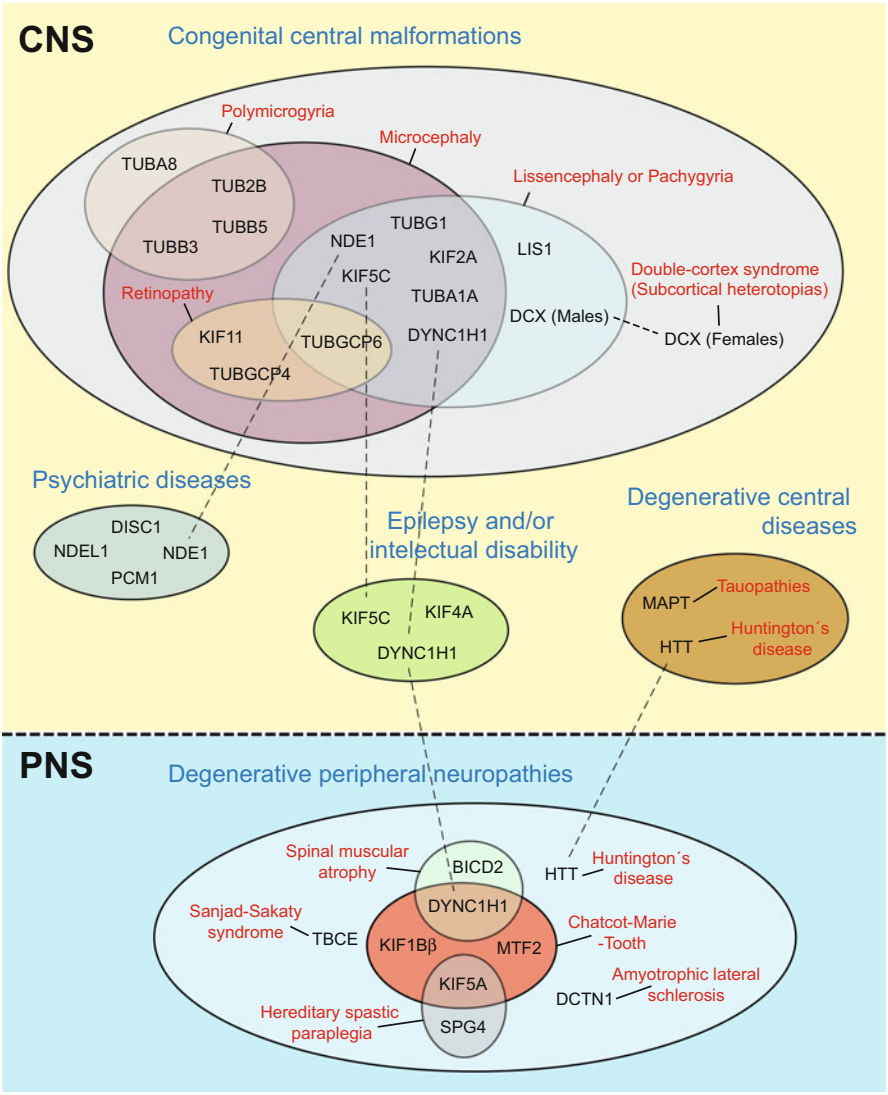

Fig. 4.3 Genes encoding components of the microtubule cytoskeleton and their association with nervous system diseases. The *yellow box* contains genes linked to malformations or diseases of the central nervous system (*CNS*). The *blue box* below contains genes linked to syndromes of the peripheral nervous system (*PNS*). Gene names (*black font*) are grouped within ovals according to the specific malformations or diseases that they have been linked to (*red font*). These are further grouped according to their more generic classification (*blue font*). Some genes are grouped with more than one type of syndrome or malformation within a generic classification. *Black dashed lines* indicate genes that have been associated with defects under more than one generic classification

An important player in this process is the plus end-directed motor KIF23/CHO1/MKLP1. KIF23 is expressed in rat sympathetic and cortical neurons and accumulates in dendrites, being almost absent from the axon. Depletion of KIF23 causes dendrites to develop abnormally, displaying a thin and elongated axon-like morphology and axon-like organelle composition. In these dendrites the nonuniform microtubule polarity was disrupted. Minus-end-distal microtubules were drawn back to the soma, while plus end-distal microtubules were pushed forward in the dendrite. Thus, KIF23 is essential for establishment and maintenance of dendritic identity (Sharp et al. 1997; Yu et al. 2000; Barkovich et al. 2005; Lin et al. 2012). Similar results were obtained for depletion of kinesin-12 (Lin et al. 2012). Moreover, Eg5 depletion was found to decrease dendrite length and width and increase the percentage of minus end-distal microtubules in dendrites suggesting that Eg5 limits transport of minus end-leading microtubules into dendrites (Wang and Brown 2002; Baas and Mozgova 2012; Lin et al. 2012; Kahn et al. 2015).

Finally, cytoplasmic dynein can influence dendritic morphology as well, as changes in the levels of dynein cofactors NudE or Lis1 affect microtubule dynamics and dendrite branching in *Drosophila* (Arthur et al. 2015).

Even though the molecular details remain to be elucidated, the motor-dependent sorting of microtubules generated by centrosomal and non-centrosomal nucleation as well as by severing has emerged as a crucial mechanism to establish and maintain compartment-specific microtubule configurations in neurons.

4.2.6 Tubulin Isotypes, Posttranslational Modifications, and MAPs

Multiple mechanisms exist in cells to modulate the properties of microtubules including expression of various tubulin isotypes, posttranslational modification (PTM) of tubulin, and interaction with MAPs. Here we will highlight only some of these mechanisms, focusing on those relevant to neurons.

Multiple genes encoding α- and β-tubulin exist, generating a range of tubulin molecules with subtle differences in their amino acid composition, in particular in their C-terminal tail that is exposed on the microtubule surface. It was initially suggested that the different tubulin isotypes, which frequently show tissue or development stage-specific expression, generate different types of microtubules that differ in their properties (Vale et al. 1992; Hasaka et al. 2004; Baas and Mozgova 2012). While in many cell types the isotype composition of microtubules may not be crucial, certain isotypes are specifically expressed in neurons and mutations in the corresponding genes have been linked to neurodevelopmental disorders, suggesting that certain important properties of neuronal microtubules are tubulin isotype-dependent (Joe et al. 2008). We will discuss mutations in tubulin genes and their implication in disease in more detail below.

In addition to the expression of tubulin isotypes, microtubule properties can be modulated by posttranslational modification. Modifications such as acetylation, detyrosination, or glutamylation are frequently enriched on more stable

microtubules. In neurons stable microtubules are crucial for many aspects of neuron development and function, including axon specification, neuron polarization, as well as axodendritic growth and trafficking (Witte and Bradke 2008; Janke and Kneussel 2010; Funahashi et al. 2014). However, there is currently no evidence that a particular modification directly affects the biophysical properties of microtubules. Most posttranslational modifications occur at the C-terminal tail of tubulin and it has been proposed that variability in the amount, distribution, and combination of specific modifications exposed on the microtubule lattice represents a "tubulin code" that is read by motors and MAPs, regulating their binding and function (Funahashi et al. 2014; Janke 2014).

Microtubules in neurons are known to interact with a great number of MAPs, which play a crucial role in the compartmentalization of the cell. Two well-known MAPs that display a highly polarized distribution are MAP2 and tau. MAP2 associates preferentially with dendritic microtubules, whereas tau is highly enriched on axonal microtubules (Fig. 4.2) (Dehmelt and Halpain 2005; Chew et al. 2013). Inhibition of MAP2 expression reduces neuritic growth and disorganizes microtubules in cultured neurons (Caceres et al. 1992; Tischfield et al. 2010; Cederquist et al. 2012), but mice lacking MAP2 are viable and display only a slight dendritic length reduction in hippocampal neurons (Harada 2002; Barnes et al. 2007). Only in the absence of both MAP2 and MAP1B, severe defects are observed (Teng et al. 2001; Yokota et al. 2009). MAP1B belongs to the MAP1 family and can cross-link microtubules, promoting their stability. MAP1B is localized throughout the whole neuron, accumulating in the axonal shaft and growth cone and regulating axonal growth (Mansfield et al. 1991; Black et al. 1994; Bush et al. 1996; Gonzalez-Billault et al. 2001). The protein tau also functions in microtubule stabilization. Tau has an important role in axon specification, growth, and branching (Caceres et al. 1991; Liu et al. 1999). Tau has around 80 predicted serine/threonine phosphorylation sites and many have been confirmed (Billingsley and Kincaid 1997). Increasing levels of tau phosphorylation generally result in less binding to microtubules. Phosphorylation of tau is regulated spatially and temporally during development, and hyperphosphorylation of tau is associated with disruption of the microtubule cytoskeleton and abnormal physiological events and disease, as will be discussed later in this chapter (Takashima 2013).

4.3 Defects in the Neuronal Microtubule Cytoskeleton and Disease

In the following paragraphs we will highlight examples of neurodevelopmental and neurodegenerative disorders caused by mutations in genes encoding proteins of the microtubule cytoskeleton (Fig. 4.3). Additional discussion can be found in the Chaps. 5 by Gambarotto and Basto and 6 by Theissen and Straube.

4.3.1 Mutations in α-Tubulin and β-Tubulin

Mutations in genes encoding α- and β-tubulin isotypes (TUBA1A, TUBA8 and TUBB2B, TUBB3, TUBB5) have been associated to a large spectrum of developmental brain malformations, referred to as "tubulinopathies". Patients normally suffer from microcephaly, moderate to severe motor and intellectual disabilities, and seizures. These tubulin-encoding genes are highly expressed during cortical development with specifics spatial and temporal expression patterns. In *TUBA1A*, *TUBB2B*, *TUBB3*, and *TUBB5* heterozygous missense mutations are found, whereas the unique *TUBA8* mutation consists of a homozygous 14 bp intronic deletion. The large majority of the mutations in α-tubulin and β-tubulin are predicted to impair the interaction with MAPs or motor proteins, diminish the abundance of functional tubulin heterodimers, alter GTP binding, or affect interactions within the microtubule polymer (Haque et al. 2004; Myers and Baas 2007; Tischfield et al. 2011; Bertran et al. 2011; Bahi-Buisson et al. 2014).

Since the first reports highlighting the presence of brain abnormalities related to *TUBA1A* mutations (Keays et al. 2007; Poirier et al. 2007), some common features have been consistently found in patients carrying mutations in tubulin genes. These tubulinopathy hallmarks affect mainly extra-cortical structures and include a dysmorphic aspect of the basal ganglia, the agenesis of the corpus callosum and the brainstem, and a mild to severe cerebellar hypoplasia. Together with these common extra-cortical features, specific cortical dysplasias have been linked to mutations in particular tubulin genes. These are likely the result of differences in the spatiotemporal expression patterns of the different tubulin genes (Poirier et al. 2013; Bahi-Buisson et al. 2014).

TUBA1A mutations are typically associated to type I lissencephaly, which is characterized by a smooth brain surface, absence of hypoplastic gyri and sulci, variable cortical thickness, and abnormal lamination patterns. A significant proportion of mutations in *TUBA1A* is linked to a more severe pattern of microlissencephaly. *TUBB2B* mutant patients, in contrast, show asymmetric bilateral polymicrogyria (PMG), a defect characterized by multiple small, partially fused gyri separated by shallow sulci that produce an irregular cortical surface. Neuropathological analyses revealed a disorganized layering of cortical hemispheres, the presence of ectopic clusters of neurons and heterotopias in the white matter, and an important disorganization of the radial processes of the radial glial cells (Yoon et al. 2005; Myers and Baas 2007; Jaglin and Chelly 2009; Tischfield et al. 2011; Nadar et al. 2012). *TUBB3* mutations cause a more diffuse pattern of malformations, encompassing polymicrogyria-like cortical dysplasia and ophthalmological and peripheral nerve pathologies (Tischfield et al. 2010; Poirier et al. 2013). *TUBB5* tubulinopathies show typically milder cortical dysgenesis and some reminiscent features of *TUBB3* mutants (Sharp et al. 1995, 1997; Yu et al. 2000; Liu et al. 2010, 2012; Breuss et al. 2012; Bahi-Buisson et al. 2014). The homozygous deletion on *TUBA8* is associated with polymicrogyria and corpus callosum and optic nerve hypoplasia (Abdollahi et al. 2009; Yau et al. 2014; Nguyen et al. 2014).

The tubulin-related cortical dysgenesis is thought to be caused by a combination of abnormal neuron proliferation, migration and differentiation, and axon growth and guidance defects (Yuba-Kubo et al. 2005; Jaglin and Chelly 2009; Tischfield et al. 2011). Proliferative abnormalities and their linkage to microcephaly are described in detail in the Chap. 5 by Gambarotto and Basto. Since an altered neuron migration process may explain the abnormal lamination phenotypes such as lissencephaly or heterotopias, the tubulin-related cortical dysgeneses were primarily classified as neuronal migration disorders (Barkovich et al. 2005). Supporting this idea, investigations carried out on TUBA1A-deficient mice or by knocking down TUBB2B expression in rodent embryos have shown that TUBA1A and TUBB2B are necessary for proper radial neuron migration (Keays et al. 2007; Jaglin and Chelly 2009; Lin et al. 2012). Thus the specific tubulin isotype composition of neuronal microtubules is crucial for their function in neuron migration.

In addition to lamination defects, tubulinopathies progress with dysgenesis of extra-cortical axon tracts such as the internal capsule or the corpus callosum. The anomalies in the corpus callosum, internal capsule, and other nerve tracts observed in a subset of *TUBB3* mutant patients have been interpreted as resulting from axon growth and/or guidance defects (Chew et al. 2013). Moreover, inherited missense mutations in *TUB2B* and *TUBB3* genes assayed in mice models revealed axon guidance defects and dysinnervation without evidence of neuronal proliferation or migration abnormalities (Tischfield et al. 2010; Cederquist et al. 2012; Scheidecker et al. 2015). Other studies have shown that the growth and guidance of axon bundles projected by the cortical pyramidal neurons strongly depend on microtubule regulator proteins. For instance, the genetic deletion of the Ser/Thr kinase LKB1, a key activator of several MAPs involved in neuron polarization and morphogenesis, prevents specifically the formation of the axons of the internal capsule and the corpus callosum (Pilz et al. 1998; Barnes et al. 2007). Also, targeting the adenomatous polyposis coli (APC) protein, which binds to MT plus ends, impairs the formation of the major post-migratory cortical and extra-cortical axon tracts (Yokota et al. 2009; Reiner 2013).

4.3.2 Mutations in γTuRC Subunits

Heterozygous missense mutants and allele variants in genes encoding different subunits of the microtubule nucleator γTuRC (TUBG1, TUBCGP4, and TUBGCP6) have been recently linked to brain malformations (Puffenberger et al. 2012; Poirier et al. 2013; Martin et al. 2014; Scheidecker et al. 2015; Arthur et al. 2015). Cortical dysgenesis related to *TUBG1* mutations consists mainly of severe microcephaly combined with a classic lissencephaly and predominant posterior pachygyria. Interestingly, the few patients analyzed had a normal brainstem and cerebellum formation (Sapir et al. 1997; Poirier et al. 2013). Neuron migration defects are likely a major contributor to the pathology in TUBG1 patients, since TUBG1 knockdown leads to neuronal migratory defects in mice (Wynshaw-Boris et al. 2010; Vallee et al. 2012; Poirier et al. 2013). However, considering that

γ-tubulin is central to the function of the nucleator γTuRC, other major defects in neuronal morphogenesis and function can be expected (Vallee and Tsai 2006; Yau et al. 2014; Nguyen et al. 2014). Mammals have two γ-tubulin genes, which encode the two highly similar γ-tubulin proteins TUBG1 and TUBG2 (>97 % identical). In mice, deficiency of the ubiquitously expressed TUBG1 is embryonically lethal, whereas loss of TUBG2, which is predominantly expressed in the brain, does not cause major histological or behavioral abnormalities (Tanaka et al. 2004; Yuba-Kubo et al. 2005; Umeshima and Hirano 2007; Tsai et al. 2007).

Patients carrying *TUBGCP6* mutations show microcephaly with diffuse cortical pachygyria, hypoplastic cerebellum, reduced axonal tracts in the corpus callosum, and retinopathy (Puffenberger et al. 2012; Martin et al. 2014). *TUBGCP4* allele variants associate with autosomal recessive microcephaly and retinopathy as well. A more detailed analysis of fibroblasts from mutant-*TUBGCP4* patients showed reduced γTuRC levels, altered microtubule nucleation and organization, abnormal cell morphology, and mitotic defects (Reiner 2013; Scheidecker et al. 2015). These studies establish that the γTuRC is an important regulator of brain development.

4.3.3 Mutations in MAPs

Mutations in genes encoding MAPs have been associated to severe central and peripheral neuropathies. Mutations in the lissencephaly-1 (*LIS1*) and doublecortin (*DCX*) genes account for a majority of the cases of lissencephaly syndrome (Pilz et al. 1998; Moores et al. 2004; Bechstedt and Brouhard 2012). However, some differences have been noted between patients. Whereas mutations in *LIS1* are found mostly in patients with type I lissencephaly affecting primarily the dorsal part of the brain, mutations in *DCX* primarily affect the rostral regions of the brain and are the major cause of the X-linked lissencephaly, also termed double cortex syndrome (Tanaka et al. 2006; Koizumi et al. 2006; Tint et al. 2009; Jean et al. 2012; Reiner 2013).

LIS1 is an atypical MAP which can modulate microtubule dynamics and organization in mammalian cells (Sapir et al. 1997). LIS1 interacts with the proteins NDE1 and NDEL1 for regulation of dynein-driven cell motility (Wynshaw-Boris et al. 2010; Vallee et al. 2012). Genetic ablation of *Lis1* in mice results in peri-implantation lethality, but the usage of hypomorphic alleles and *in utero* electroporation of siRNAs have shown that LIS1 plays a critical role in neuron migration during development. The migration deficits observed lead to a disorganized layering of the neocortex, hippocampus, cerebellum, and olfactory bulb, which subsequently interfered with normal cognition and motor coordination (Vallee and Tsai 2006). More detailed studies revealed that LIS1 deficiency specifically impairs the nuclear translocation during neuron migration without interfering with the coupling of microtubules and centrosome in the leading process (Tanaka et al. 2004; Umeshima and Hirano 2007; Tsai et al. 2007).

DCX is a phospho-MAP expressed in migrating and differentiating mammalian neurons during the period of corticogenesis. It can be phosphorylated by a number

of kinases such as JNK, Cdk5, PKA, or GSK3b, controlling its affinity for microtubules (Pramparo et al. 2010; Reiner 2013). DCX interacts with and stabilizes the lattice of microtubules. Interestingly, mutations in *DCX* found in patients with double cortex syndrome disrupt this mechanism (Wieczorek et al. 1999; Fonknechten et al. 2000; Parvari et al. 2002; Moores et al. 2004; Bechstedt and Brouhard 2012). DCX contributes to many aspects of brain development, including axonal and dendritic arborization, microtubule organization in growth cones, and the maintenance of bipolar morphology during neuron migration (Parvari et al. 2002; Tanaka et al. 2006; Koizumi et al. 2006; Tint et al. 2009; Jean et al. 2012). DCX-deficient mice show normal cortical lamination and no gross neurodevelopmental defects, likely due to compensation. Indeed, double knockout of *Dcx* and *Dclk1*, a doublecortin-like kinase, revealed neuron migratory deficits producing abnormalities in the neocortical and hippocampal lamination (Tanaka et al. 2006; Kappeler et al. 2006). Interestingly, LIS1 and DCX have overlapping localization and coimmunoprecipitate in brain lysates and DCX can rescue centrosome-nucleus uncoupling and neuron migration defects triggered by LIS1 or dynein loss-of-function (Martin et al. 2002; Tanaka et al. 2004; Schaefer et al. 2007; Jin et al. 2009).

Although multiple evidences indicate that mutations in *LIS1* and *DCX* impair neuronal migration, neurogenesis deficits could also contribute to the pathology in affected patients. Both LIS1 and DCX participate in the regulation of neuron proliferation in the developing brain by influencing mitotic spindle orientation of neuroepithelial stem cells and radial glia progenitors or by acting in the interkinetic motility of the radial glia (Tsai et al. 2005; Beetz et al. 2006; Pramparo et al. 2010).

Other microtubule-interacting proteins associated with neurological and developmental defects are the tubulin-specific chaperone TBCE and the microtubule-severing protein spastin (Wieczorek et al. 1999; Fonknechten et al. 2000; Parvari et al. 2002; Liu et al. 2009; Deluca et al. 2015). Deletion and truncation mutations in *TBCE* were identified in patients with congenital hypoparathyroidism, mental retardation, facial dysmorphism (HRD or Sanjad-Sakaty syndrome) (Parvari et al. 2002; Fink and Rainier 2004; Tarrade et al. 2006). TBCE protein is critical for microtubule maintenance in mouse motor axons, and its down-regulation correlates with peripheral axon retrograde degeneration (die back process) and developmental defects in neuromuscular synapses (Weingarten et al. 1975; Drechsel et al. 1992; Martin et al. 2002; Schaefer et al. 2007; Jin et al. 2009). Mutations in the spastin gene (*SPG4*) are responsible for 40 % of autosomal dominant forms of hereditary spastic paraplegia (HSP), also called the "dying-back" neuropathy (Beetz et al. 2006; Elie et al. 2015). The major clinical feature of this disease is gait disturbance with muscle spasticity and weakness, seemingly due to axon loss in motor and sensory tracts and to the presence of axon swellings in patients (Liu et al. 2009; Tenreiro et al. 2014; Deluca et al. 2015). Mice models homozygous for *Spg4* mutations reproduce these degenerative hallmarks and show deficits in axonal trafficking. Although it was suggested that *SPG4* mutations alter the interaction with microtubules, the microtubule pathology underlying the axonal

phenotypes is not completely understood (Fink and Rainier 2004; Tarrade et al. 2006; Hernández and Avila 2007; Beharry et al. 2014).

Tau (MAPT) is another neuronal MAP strongly associated with a long list of neurodegenerative dementias. It is found preferentially bound to axonal microtubules, and it can promote microtubule polymerization and stability (Weingarten et al. 1975; Drechsel et al. 1992; Taymans et al. 2014; Vuono et al. 2015). Interestingly, a recent study shows tau binding simultaneously to actin and microtubules in vitro, coordinating a coupled growth of both networks (Zhang et al. 2014; Elie et al. 2015). Tau is a phosphoprotein with multiple phosphorylation sites and is a substrate of at least 20 protein kinases, including GSK3b, CDK5-p25, MARK, or PKA (Millecamps and Julien 2013; Tenreiro et al. 2014). The accumulation in brain tissue of abnormally hyperphosphorylated tau protein as filamentous aggregates is a common feature of several dementias, collectively referred to as tauopathies. This family of diseases includes Alzheimer's disease (AD), frontotemporal dementia (FTD) with or without parkinsonism-17, corticobasal degeneration (CBD), Pick's disease, progressive supranuclear palsy (PSP), or dementia pugilistic (Hernández and Avila 2007; Iqbal et al. 2009; Combs and Gamblin 2012; Beharry et al. 2014). Moreover tau pathology has been recently associated also with Huntington's and Parkinson's disease (Ishihara et al. 1999; Zhang et al. 2004; Taymans et al. 2014; Vuono et al. 2015).

Six tau isoforms are produced by alternative splicing in the adult human brain. At least 59 mutations have been found in exons and introns, mostly associated to FTDP-17, PSP, Pick's disease, and CBD. Many of these disease-related tau mutations promote the exon 10 inclusion in the spliced variants, resulting in the disruption of the tau isoform balance and triggering pathology (Zhang et al. 2014). Hyperphosphorylated tau has a reduced affinity for microtubules, lowering the ability to promote microtubule assembly and stabilization and influencing motor transport along microtubules (Millecamps and Julien 2013). Some of the pathogenic tau mutations were shown to make the protein more easily abnormally phosphorylated, inducing protein aggregation and microtubule-related dysfunctions (Iqbal et al. 2009; Combs and Gamblin 2012). To address the physiological consequences, transgenic mice overexpressing wild type or mutant tau were generated. These animals present axonal transport deficits and axon swellings preceding the appearance of tau protein aggregates, which could explain the observed synaptic deficits and neurodegeneration, and the associated cognitive symptoms (Ishihara et al. 1999; Zhang et al. 2004).

4.3.4 Mutations in Motors and Motor-Associated Proteins

Abnormalities in the intracellular transport machinery are considered risk factors for a wide variety of both central and peripheral congenital or degenerative diseases. Most of the neurodegenerative diseases present axon pathology and accumulation of aggregates of certain microtubule-related proteins in different neuron types, associated with axonal trafficking defects. However, the causal

relationship between transport impairments and neurodegeneration is currently unclear. In addition to diseases associated with abnormal protein aggregation, some neuropathies have been linked to mutations in motor or motor-associated proteins in the absence of any protein aggregates (Mansfield et al. 1991; Black et al. 1994; Takashima 2013; Millecamps and Julien 2013). Examples will be discussed in the following sections.

4.3.4.1 Dynein

A missense mutation in the homodimerization domain of the heavy-chain DYNC1H1 of the dynein motor complex has been identified in a form of Charcot-Marie-Tooth (CMT) disease, which is characterized by axonal degeneration with distal sensory loss and weakness (Weedon et al. 2011). Mice carrying mutations in this same domain show impaired axonal retrograde transport and age-related progressive loss of muscle tone and locomotor skills (Hafezparast et al. 2003; Chen et al. 2007). CMT disease is also associated with mutations in other genes including *MTF2*, which encodes a mitochondrial protein that interacts with adaptor proteins of kinesin motors. Accordingly, *Mtf2* knockout mice display defects in mitochondrial motility (Züchner et al. 2004; Cartoni et al. 2010).

Mutations in *DYNC1H1* have also been linked to spinal muscular atrophy (SMA), a disease of the peripheral nervous system impairing muscle movement and leading to muscle weakening, and to developmental cortical malformations and severe intellectual disability in the central nervous system (Willemsen et al. 2012; Poirier et al. 2013; Jamuar et al. 2014). Recessive hypomorphic variants of the heavy-chain *DYNC2H1* of the dynein complex are responsible for some human ciliary disorders (Huber and Cormier-Daire 2012). Moreover, genetic mutations in the dynein regulator *BICD2* have been found in patients with dominant congenital SMA (DCSMA), characterized by nonprogressive congenital early-onset lower-limb-predominant weakness. BICD2 participates in the transport of RAB6 vesicles and other dynein-dependent trafficking, and some pathogenic *BICD2* mutations have shown to alter RAB6 binding and produce Golgi fragmentation (Oates et al. 2013; Peeters et al. 2013; Rossor et al. 2015).

Mutations in the gene *DCTN1*, which encodes the $p150^{glued}$ subunit of the dynein cofactor complex dynactin, are associated with amyotrophic lateral sclerosis (ALS), a progressive neurodegenerative disease caused by functional impairment and degeneration of motor neurons in the brain and the spinal cord (Puls et al. 2003; Münch et al. 2004; Stockmann et al. 2013). Supporting a role of dynactin in ALS, *DCTN1* expression was found to be strongly downregulated in sporadic ALS patient samples (Jiang et al. 2005). However, no other clear links to ALS were established for other DCTN1 genetic variations (Farrer et al. 2009).

Mutations in other dynein adaptors have also been linked to disease. NDE1 regulates mitotic spindle assembly in cortical progenitors, whereas NDEL1 controls the microtubule-dependent coupling of centrosome and nucleus in migrating postmitotic neurons (Feng and Walsh 2004; Shu et al. 2014). Only mutations in the *NDE1* gene have been associated to developmental cortical malformations, such as microcephaly with a spectrum of lissencephaly (referred to as

microlissencephaly) and fetal brain disruption. The pathological NDE1 versions showed impaired centrosomal localization and altered binding to cytoplasmic dynein (Alkuraya et al. 2011; Bakircioglu et al. 2011; Paciorkowski et al. 2013). However, both *NDE1* and *NDEL1* have been identified in genetic association studies of mental illnesses, linked to risk variants of another dynein adaptor, DISC1 (Bradshaw and Porteous 2012). DISC1 (Disrupted In Schizophrenia 1) protein is implicated in embryonic and adult neurogenesis, radial and tangential modes of neuron migration, and synaptic function. Together *DISC1*, *NDE1*, and *NDEL1* regulate the functions of the microtubule network in cortical development and neurite formation and mutation of the corresponding genes is a robust genetic risk factor for a wide range of psychiatric disorders (Thomson et al. 2013; Lipina et al. 2013). The microtubule-associated protein PCM1, which participates in protein recruitment to the centrosome and influences centrosomal microtubule organization, is a DISC1 interactor and has been also genetically associated with schizophrenia (Zoubovsky et al. 2015).

Importantly, dynein is also involved in the clearing of abnormal protein aggregates by autophagy. Autophagy is a catabolic neuroprotective mechanism ensuring the constant removal of damaged organelles or proteins. The targeted materials are engulfed by autophagosomes, which are then fused to endosomes and lysosomes for the final degradation of their content. Whereas in healthy neurons autophagosomes move in the axons mostly retrogradely through dynein-dependent transport (Yang et al. 2013), accumulation of autophagy-related vesicles is frequently observed in PD, AD, or HD samples. Dynein-dependent vesicular transport deficits are believed to contribute to defective autophagy and thus to axonal degeneration (Chen et al. 2012; Wong et al. 2015).

4.3.4.2 Kinesins

Most members of the kinesin superfamily drive microtubule plus end-directed transport, which in axons permits anterograde cargo movement toward the nerve terminals.

Mutations in the neuron-specific kinesin-1 family member *KIF5A* are responsible for dominant forms of hereditary spastic paraplegia (HSP), a group of diseases showing progressive spasticity in the lower limbs. The defects are thought to be caused by a reduction in KIF5A microtubule affinity and thus in transport activity (Ebbing et al. 2008; Goizet et al. 2009). A mutation in *KIF5A* has also been linked to Charcot-Marie-Tooth disease type 2 (CMT2) (Crimella et al. 2012). In other CMT2 patients, however, a loss-of-function mutation in the motor domain of the kinesin-3 family member *KIF1Bbeta* was identified (Zhao et al. 2001).

Missense genetic variants of the kinesins KIF2A and KIF5C have been identified in individuals with microcephaly and cortical malformations. Whereas the mutation in *KIF5C* seems to impair the protein's ability to hydrolyze ATP, the pathogenic *KIF2A* mutation causes protein misfolding and loss-of-function (Poirier et al. 2013; Jamuar et al. 2014).

Mutant versions of *KIF5C* and also *KIF4A* have been identified as risk factors for intellectual disability and epilepsy, due to an imbalance between excitatory and inhibitory synaptic activity (Willemsen et al. 2014).

Heterozygous mutations in the homotetramer kinesin motor Eg5/KIF11, a protein implicated in mitotic spindle assembly and with neuronal functions in axonal path finding and dendrite morphogenesis, cause microcephaly, lymphedema, chorioretinopathy, and retinal detachment (Ostergaard et al. 2012; Jones et al. 2014; Robitaille et al. 2014).

Huntingtin (HTT), a multi-domain protein with multiple but poorly understood cellular roles, interacts direct and indirectly (through its partner HAP1) with kinesin light chain 1 (KLC1), the p150 subunit of dynactin and with dynein intermediate chains. It has been proposed that HTT acts as a molecular switch: when it is phosphorylated, HTT associates with kinesin-1 to promote anterograde transport, and when it dephosphorylates, kinesin-1 dissociation favors dynein-dependent retrograde transport (Colin et al. 2008). However, the precise molecular mechanism is not completely understood. The expansion of CAG codon repetitions in the coding region of the *HTT* gene is cause of Huntington's disease (HD), an adult-onset autosomal dominant neurodegenerative disease that impairs muscle coordination and leads to cognitive decline and dementia. The expansion of the CAG repetitions generates an extended poly-glutamine region in the HTT protein, which alters HTT function, induces abnormal HTT aggregates, and ultimately leads to the degeneration of striatal and cortical neurons (Gil et al. 2008; Caviston et al. 2009).

4.4 Conclusion

The wide range of neurodevelopmental and degenerative diseases associated with defects in the microtubule cytoskeleton reveals that neurons are particularly sensitive to perturbances affecting microtubule organization and/or function. Despite this fact, our understanding of how the neuronal microtubule network is assembled and maintained, and how its different components function at the molecular level, is still very limited. Addressing these issues in the future will not only be crucial for uncovering disease mechanisms, a prerequisite for developing therapeutic strategies, but will also be informative for understanding microtubule organization and function in other cell types.

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Consequences of Numerical Centrosome Defects in Development and Disease

5

Davide Gambarotto and Renata Basto

Abstract

Defects in centrosome number or structure can have considerable consequences for the physiology of an organism. Aberrant centrosome number has been proposed for a century to contribute to genome instability and tumour formation. However, in the last decade, mutations in centrosome genes have been described in diseases characterised by defective growth. Centrosome dysfunction can therefore have opposite effects on the homeostasis of the organism. Here we discuss how deregulation of centrosome number during embryonic development might contribute to growth defective syndromes such as autosomal recessive primary microcephaly (MCPH) and primordial dwarfism. We further discuss how the same defects might play a role in cancer when present in adult tissues.

5.1 Introduction

The centrosome is the major microtubule-organising centre of animal cells (Kellogg et al. 1994). It participates in different processes such as cell division, motility and polarity, mainly by organising the microtubule network. Centrosomes are not present in plants, whereas fungi have an analogous structure called the spindle pole body (Marshall 2009).

The centrosome is composed by two centrioles surrounded by the pericentriolar material (PCM) (Nigg and Raff 2009) (for a discussion of PCM structure and function, see also the Chap. 3 by Comartin and Pelletier). Centrioles are cylindrical structures made of nine microtubule triplets arranged in a ninefold symmetry. They recruit and organise a large number of proteins forming the PCM (Bobinnec et al. 1998).

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Centriole number is tightly regulated. For most part of the cell cycle, the two centrioles are linked and placed orthogonally to each other. This configuration is called centriole engagement, and a new centriole is not formed as long as the pre-existing centrioles are engaged (Tsou and Stearns 2006). Centriole disengagement takes place usually at the end of mitosis when the daughter cells inherit one centrosome with two separated centrioles (Kuriyama and Borisy 1981). Centriole disengagement is thought to be the licence to allow centriole duplication (Tsou and Stearns 2006), which occurs only once per cell cycle.

Five proteins, ZYG-1, SPD-2, SAS-4, SAS-5 and SAS-6, were identified in *Caenorhabditis elegans* as essential for centriole biogenesis (Dammermann et al. 2004; Delattre et al. 2004; Kemp et al. 2004; Kirkham et al. 2003; Leidel et al. 2005; Leidel and Gonczy 2003; O'Connell et al. 2001; Pelletier et al. 2004). These proteins are recruited in a precise temporal order (Delattre et al. 2006; Pelletier et al. 2006). SPD-2 is the first to be recruited to the parental centriole, which in turn is required for ZYG-1 recruitment. Shortly after, a complex conteining SAS-5 and SAS-6 is recruited to structurally form the centriole. Finally SAS-4 is recruited to allow the incorporation of microtubules into the centriole wall.

Remarkably, all these genes have a sequence or a functional ortholog in most animals (Carvalho-Santos et al. 2010; Hodges et al. 2010). Human PLK4, also called SAK in *Drosophila*, is the functional ortholog of ZYG-1 and, like ZYG-1, is a serine-threonine kinase (Bettencourt-Dias et al. 2005; Habedanck et al. 2005) (Fig. 5.1). CEP192 is the human ortholog of SPD-2 (Andersen et al. 2003; Pelletier et al. 2004). SAS-4 orthologs are called CPAP in humans (Tang et al. 2009) and DSas-4 in *Drosophila* (Basto et al. 2006). STIL and Ana2 are, respectively, the human and the *Drosophila* functional orthologs of SAS-5 (Arquint et al. 2012; Stevens et al. 2010; Tang et al. 2011; Vulprecht et al. 2012). SAS-6 is called HsSAS-6 in humans and DSas-6 in flies (Gopalakrishnan et al. 2010; Leidel et al. 2005; Peel et al. 2007; Rodrigues-Martins et al. 2007a; Strnad et al. 2007).

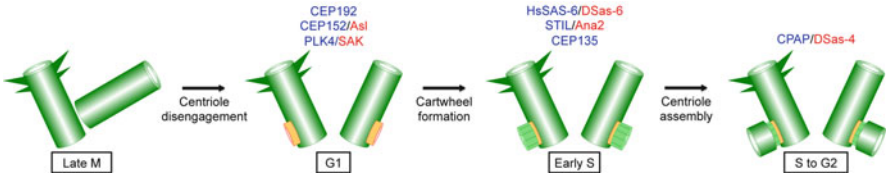

Fig. 5.1 The centriole duplication cycle. For most part of the cell cycle, the two centrioles (*dark green cylinders*) are linked and placed orthogonally to each other. This configuration is called centriole engagement. At the end of mitosis, each daughter cell inherits one centrosome with two separated centrioles (centriole disengagement). This event is permissive to centriole duplication. Centriole duplication is initiated when CEP192 and CEP152/Asl recruit PLK4/SAK at the proximal end of the mother centriole (*yellow ring*). PLK4/SAK activity is required for cartwheel formation (*light green ring*). The cartwheel is composed of SAS-6, STIL/Ana2 and CEP135 and is the first visible structure of the new centriole. Subsequently, SAS-4 is recruited to allow the incorporation of nine sets of microtubules (centriole assembly). During G2 the procentriole (*short dark green cylinders* at the proximal end of the mother centriole) elongates to achieve the size of the mother

Similarly to their orthologs in *C. elegans*, centriolar recruitment of these proteins is hierarchically regulated (Kleylein-Sohn et al. 2007). PLK4/SAK is the master regulator of centriole duplication, and in human cells, it is recruited to the mother centriole by CEP192 in collaboration with another protein not present in *C. elegans* called CEP152 (Kim et al. 2013; Sonnen et al. 2013). In *Drosophila*, SPD-2 is dispensable for centriole duplication (Dix and Raff 2007; Giansanti et al. 2008) and SAK recruitment is entirely fulfilled by the CEP152 ortholog, asterless (Asl) (Dzhindzhev et al. 2010). PLK4/SAK triggers centriole formation through direct interaction with STIL/Ana2, and its phosphorylation by PLK4/SAK (Dzhindzhev et al. 2014; Kratz et al. 2015; Ohta et al. 2014) is then required for centriolar loading of HsSAS-6/DSas-6. STIL/Ana2 and HsSAS-6/DSas-6 form the cartwheel, which is the first identifiable structure in pro-centriole assembly (Guichard et al. 2010; Kitagawa et al. 2011b; Kuriyama 2009; van Breugel et al. 2011). Subsequently, CPAP is recruited and, according to cell type, nine sets of microtubule (Kleylein-Sohn et al. 2007) singlets, doublets or triplets will complete the centriole structure (Fig. 5.1). Although in humans extra proteins participate in procentriole assembly, such as CEP135, CP110 and γ-tubulin (Kleylein-Sohn et al. 2007), the core duplication machinery is well conserved through evolution (Carvalho-Santos et al. 2010; Hodges et al. 2010) (see Fig. 5.1 and Chap. 3 for further details on centriole duplication).

5.2 Animals Without Centrosomes

In 1887, Van Beneden and Boveri described the centrosome as "the organ for cell division". This statement was justified by the presence of centrosomes at the spindle poles, suggesting a function in spindle formation. For this reason, it has long been accepted that centrosomes were essential for cell division. However, the discovery that many eukaryotic organisms, such as plants, do not have centrosomes suggested that probably centrosomes are dispensable to form a bipolar spindle, at least in some cell types. Indeed, many cells form a bipolar spindle and divide even in absence of centrosomes. One interesting example is the mouse embryo that forms "acentrosomal" spindles in the first cleavages (Szollosi et al. 1972). Other examples are female oocytes from most animal species that assemble "acentrosomal" spindles during both meiotic divisions (Schatten 1994). In the absence of centrosomes, microtubules are nucleated at the vicinity of the chromatin, and with the help of molecular motors, spindle poles become focused (Heald et al. 1996; Karsenti et al. 1984; Khodjakov et al. 2003; Maiato et al. 2004). In addition to this pathway, microtubules can also be generated within the mitotic spindle from pre-existing microtubules. In this case, γ-tubulin is required and its localisation depends on the augmin complex (Goshima et al. 2008).

All these pathways exist in cells that form mitotic spindle via the "classical" centrosome-dependent mechanism, and the three collaborate to assemble a functional bipolar spindle that accurately segregates chromosomes (Meunier and Vernos 2012).

Even if some cell types in animals can undergo cell division in the absence of centrosomes, it was difficult to imagine that an entire organism could develop without centrosomes. In a surprising study, it was shown that the planarian *Schmidtea mediterranea* does not contain centrosomes at any stage of its life cycle (Azimzadeh et al. 2012). Interestingly, planarians need to assemble cilia in some cell types, and *de novo* centriole formation in these cells ensures the presence of basal bodies for ciliogenesis. PCM proteins such as SPD-2/CEP192 and Cnn/CDK5RAP2 are absent from *S. mediterranea,* and cell division does not depend on centrosomes. Since centrosomes also play important roles in spindle positioning during oriented cell divisions (Morin and Bellaiche 2011), these results also put in evidence that regeneration, a process frequently used in planaria, does not require centrosome-dependent spindle positioning (Cardona et al. 2006).

But what happens if centrosomes are removed from somatic cells that normally contain centrosomes? This question was initially addressed in vertebrate cells where centrosomes were removed by laser ablation or microsurgery (Hinchcliffe et al. 2001; Khodjakov et al. 2000). In these cells, a bipolar spindle, which could correctly segregate chromosomes within the following cell division, was assembled. Recently however, it has been shown that permanent removal of centrosomes in chicken DT40 cells by knockout of either *CEP152* or *STIL* results in abnormal chromosome segregation (Sir et al. 2013). In these cells, the spindle was disorganised explaining probably the high rate of segregation errors observed. The authors proposed that organisms with high number of chromosomes, such as chicken, which has 78 chromosomes, strongly rely on centrosome-driven spindle assembly for mitotic fidelity. It is important to mention, however, that these cells are non-adherent and might require centrosomes for mitotic spindle assembly while other cell types that undergo mitosis while adhering to a substrate do not (Hinchcliffe et al. 2001; Khodjakov et al. 2000).

Flies that carry mutations in the centriole duplication genes *asl*, *DSas-4* and *PLK4*/*SAK* undergo larval development giving rise to adults without any morphological defect (Basto et al. 2006; Bettencourt-Dias et al. 2005; Blachon et al. 2008). Importantly, this is possible because maternally provided components ensure centriole duplication at early developmental stages. In the absence of centrosomes, early embryonic development is impaired and embryos arrest during syncytial stages (Stevens et al. 2007). The centrosome-dependent spindle assembly mechanism is probably extremely important during the rapid mitotic cycles occurring after fertilisation, and astral microtubules are required for nuclear separation after anaphase in the preblastoderm cytoplasm (Telley et al. 2012).

Acentriolar flies also lack cilia and flagella, which are essential in *Drosophila* for adult viability and male fertility, respectively. In flies very few cells contain cilia. Type I mechanosensory neurons are ciliated, and adults that lack centrioles are severely uncoordinated and die a few hours after eclosion (Baker et al. 2004; Dubruille et al. 2002; Gogendeau and Basto 2010; Martinez-Campos et al. 2004). Another cell type that requires centrioles for cell division in flies are primary spermatocytes that normally have long centrioles containing microtubule triplets. In the absence of centrioles, meiotic spindles present broad poles and are highly

disorganised giving rise to unviable aneuploid progeny (Basto et al. 2006; Bettencourt-Dias et al. 2005; Martinez-Campos et al. 2004; Rodrigues-Martins et al. 2007b).

Centrosomes, through astral microtubule nucleation, also contribute to accurate spindle positioning. This is particularly important when cell fate determinants should be segregated into daughter cells during asymmetric and even symmetric divisions (Knoblich 2008). *Drosophila* acentriolar larval brain stem cells (NSCs) divide symmetrically and give rise to two stem cells, at the expense of differentiating cells (Basto et al. 2006; Giansanti et al. 2001; Megraw et al. 2001), which causes tumour formation in transplantation assays (Castellanos et al. 2008). Centrosomes also participate in spindle positioning in *Drosophila* male germline stem cells (Yamashita et al. 2003); however, other mechanisms contribute to tissue homeostasis in the absence of centrosomes (Riparbelli and Callaini 2011; Sheng and Matunis 2011). In female germline stem cells however, centrosomes are dispensable and spindle positioning depends on the function of the fusome (Stevens et al. 2007), a membrane skeleton-enriched structure typical of the insect germline (Lin et al. 1994).

In vertebrates, most cells contain a primary cilium, and centriole loss has severe consequences during development. Embryos lacking cilia have defective body plan organisation caused by disruption of hedgehog signalling pathway and arrest at E10.5–11.5 (Goetz and Anderson 2010). Recently, the developmental functions of mammalian centrioles *in vivo* have been analysed (Bazzi and Anderson 2014). Characterisation of a null *Cpap* (the *SAS-4* ortholog) mutant mouse revealed that centrosomes are essential during embryonic development. $Cpap^{-/-}$ mice died at early stages (E9.0) with increased p53-dependent cell death. Interestingly, increase in DNA damage or aneuploidy was not observed. Instead, cells displayed a prolonged prometaphase, and consequently mitosis completion was delayed, suggesting a requirement for centrosomes for rapid bipolar spindle assembly in vertebrates. Null mutant mice for other centriole duplication genes, *Plk4*, *Stil* and *Cep152*, also arrested early in development, at the same stage of the $Cpap^{-/-}$ mice, showing increased apoptosis and increased p53 levels (Bazzi and Anderson 2014; Hudson et al. 2001; Izraeli et al. 1999). Intriguingly, Bazzi and colleagues noticed that in embryos lacking centrioles, regions with higher proliferation rates showed higher p53 levels. Since these embryos died earlier than those lacking cilia (Huangfu et al. 2003), it is possible that the absence of centrioles *per se* up-regulates p53 in rapidly proliferating cells, causing widespread cell death and consequent lethality. Probably, the apoptotic pathway is triggered in cells that are not able to go through mitosis as fast as they should. In zebra fish, depletion of *stil* induced a similar phenotype (Pfaff et al. 2007). Apoptosis was also increased and embryos died between 7 and 10 days post-fertilisation. Spindles were monopolar or highly disorganised, which resulted in delayed mitotic progression.

Overall, these studies show an unexpected up-regulation of cell death by apoptosis in response to prolonged mitosis or mitotic arrest. It will be important in the future to determine why the apoptotic pathway is triggered in the absence of centrosomes, when aneuploidy is not being generated (Bazzi and Anderson

2014). In particular it will be essential to understand how the lack of centrosomes is detected in embryonic cells and then translated into p53 up-regulation and apoptosis.

5.3 Animals with Extra Centrosomes

Differentiated cells have one centrosome that organises the microtubule network or cilia, whereas cycling cells, in mitosis, have two centrosomes to form a bipolar spindle. The presence of more than two centrosomes in a cell is called centrosome amplification, and it has been described in both physiological and pathological conditions. There are three main ways to induce centrosome amplification:

1. Cytokinesis failure: it generates tetraploid cells with four centrosomes. Cytokinesis failure occurs physiologically in certain mammalian tissues during postnatal growth such as hepatocytes (Guidotti et al. 2003).
2. Cell fusion: during development, cell fusion is involved in many processes, such as the formation of trophoblast cells in the mammalian placenta, muscles and osteoclasts (Oren-Suissa and Podbilewicz 2007). Cell fusion can also be involved in wound healing as shown in *Drosophila* larval and adult epidermis (Galko and Krasnow 2004; Losick et al. 2013).
3. Centrosome cycle deregulation: the levels of proteins involved in centriole duplication are highly regulated during the cell cycle (Marthiens et al. 2012). Their overexpression can trigger centrosome amplification (Kleylein-Sohn et al. 2007). For example, when *PLK4* is overexpressed, multiple centrioles are formed in S phase in the typical rosette-like structure surrounding the mother centriole (Habedanck et al. 2005; Kleylein-Sohn et al. 2007). At the end of mitosis, they disengage and duplicate to give rise to extra centrosomes.

The presence of extra centrosomes can induce multipolar divisions, which are in most cases not viable (Ganem et al. 2009). Cells evolved several mechanisms to enable bipolar division in the presence of centrosome amplification, and the predominant mean is the clustering of extra centrosomes in two main spindle poles (Basto et al. 2008; Kwon et al. 2008; Leber et al. 2010; Marthiens et al. 2012; Quintyne et al. 2005; Ring et al. 1982). Clustering is achieved mainly by combining spindle-intrinsic microtubule binding forces and actin-regulating forces at the cell cortex (Kwon et al. 2008). However, this mechanism can hide a threat. Extra centrosomes induce the formation of multipolar spindle intermediates during prometaphase, which promote merotelic attachments (one kinetochore attached to microtubules nucleated by different poles) and consequent chromosome missegregation during anaphase (Ganem et al. 2009; Silkworth and Cimini 2012). In certain cell types, extra centrosomes favour the nucleation of extra cilia, which leads to dilution of cilia signalling molecules such as members of the sonic hedgehog signalling pathway (Mahjoub and Stearns 2012).

Consequences of centrosome amplification at the level of the whole organism have been studied only in *Drosophila* so far. When *SAK* (the *PLK4 Drosophila* ortholog) is overexpressed in all the cells of the body, almost 60 % of somatic cells present centrosome amplification. Embryonic development is highly compromised, as clustering mechanisms are not efficient during early development (Basto et al. 2008). Larval development is slightly delayed, but adults are morphologically normal, viable and fertile. Neuroblasts (NBs) of the larval brain, which are neural stem cells, always divide in a bipolar fashion even though they harbour extra centrosomes, thanks to the very efficient centrosome clustering and inactivation mechanisms (lack of microtubule-nucleating capacity). However, in certain cases, spindles are mispositioned which results in defects in asymmetric cell division and in the generation of extra stem cells at the expense of differentiating cells. As a consequence, in transplantation assays, these brains can over-proliferate and induce tumours that kill the host prematurely (Basto et al. 2008) (see also next paragraphs). In the wing imaginal disc, the larval epithelium that gives rise to the adult wings, mechanisms of centrosome clustering and centrosome inactivation are also present but not fully efficient (Sabino et al. 2015). As a result, tripolar divisions and chromosome segregation defects occur with only minor defects in spindle positioning. This leads to aneuploid cells able to proliferate and induce tumorigenesis when transplanted into WT hosts. Hence, these studies show that centrosome amplification, at least in *Drosophila*, is a tumour-initiating event (Basto et al. 2008; Sabino et al. 2015).

The consequences of centrosome amplification in vertebrates are still not known with the exception of centrosome amplification in the mouse central nervous system (CNS) (Marthiens et al. 2013). Contrary to flies, the presence of extra centrosomes in mouse neural stem cells does not perturb spindle orientation. However, it causes aneuploidy and consequent cell death due to inefficient clustering. A major consequence of centrosome amplification in the mouse CNS is a severe reduction in brain size, a condition also known as microcephaly (see below) (Fig. 5.2).

5.4 Centrosomes and Disease

5.4.1 Centrosome Defects and Growth Failure

Generally, the number and size of cells define the size of organs and organisms (Conlon and Raff 1999). The balance between cell proliferation, differentiation and cell death contributes to determine the number of cells at the end of development (Conlon and Raff 1999). Changes in one of these parameters, in particular during embryonic development when body size is being established, can lead to growth defects (Klingseisen and Jackson 2011).

Progenitor cells undergo two different types of division: (1) to enlarge the pool of progenitors, they divide symmetrically forming two identical cells and (2) to allow differentiation, they divide asymmetrically giving rise to one progenitor cell and to another, generally more committed that will ultimately differentiate

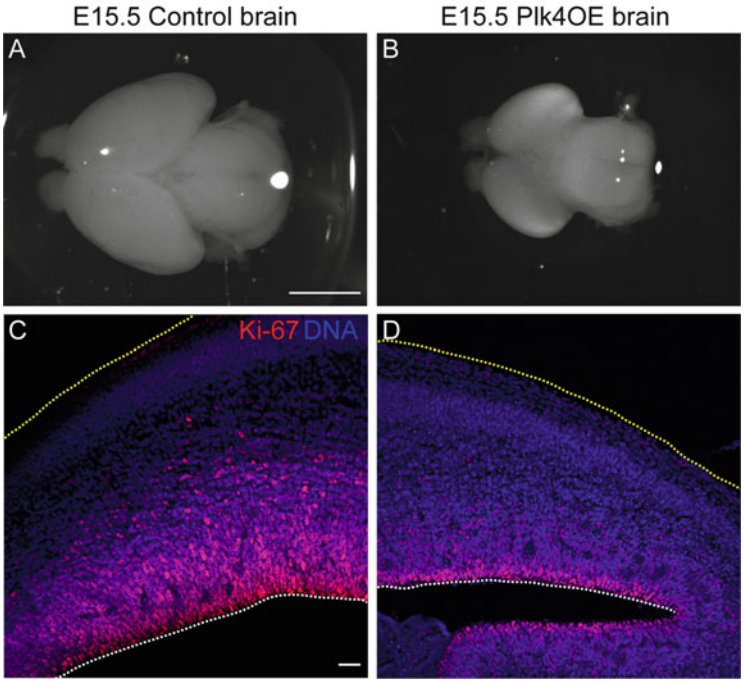

Fig. 5.2 Consequences of *Plk4* overexpression (*Plk4OE*) in the developing mouse brain. Centrosome amplification in mouse embryonic neural stem cells (NSCs) caused by the overexpression of *Plk4* results in microcephaly. Dorsal views of control (**a**) and Plk4OE (**b**) brains at E15.5. Scale bar = 2 mm. Control (**c**) and Plk4OE (**d**) E15.5 brain sections immunostained for the cycling proliferating marker Ki67 (red). DNA is shown in blue. Scale bar = 12 μm

(Tajbakhsh et al. 2009). Accurate timely control of the switch from symmetric to asymmetric division is essential to guarantee correct development and body size.

Although the mechanisms behind growth retardation during embryonic development are not entirely known, mutations in genes encoding for centrosome and centrosome-associated proteins have been reported in diseases characterised by growth deregulation, such as autosomal recessive primary microcephaly (MCPH) and the primordial dwarfism diseases Seckel syndrome (SCKS) and microcephalic osteodysplastic primordial dwarfism type II (MOPD-II).

The common feature of these syndromes is microcephaly, which is clinically defined as a smaller brain than the mean for sex, age and ethnicity, with the occipitofrontal head circumference (OFC) equal or less than -2 standard deviation (SD) (Roberts et al. 2002). In MCPH, SCKS and MOPD-II, brain structures are proportionated and present minor malformations. The phenotype of MCPH and SCKS is similar but more severe in the latter (Klingseisen and Jackson 2011). In SCKS, effects in intrauterine and postnatal growth are more pronounced. The mean OFC in children and adults is around -9 SD, whereas mean height is -7 SD. They usually suffer of mental retardation and a characteristic appearance with a narrow

and sloping forehead, prominent eyes, large and convex nose and small jaw (Hall et al. 2004; Majewski and Goecke 1982).

Stature measurement is usually the parameter used to distinguish between MCPH and SCKS: patients with normal height or between -1 SD and -2 SD are classified as MCPH, whereas those between -4 SD and -12 SD as SCKS (Verloes et al. 1993). Compared to MCPH and SCKS, in MOPD-II, growth retardation is more severe and accompanied by highly proportional reduced body size (Majewski et al. 1982). Primordial dwarfisms and MCPH are extremely rare autosomal recessive one-gene disorders, with higher incidence in populations where consanguineous marriages are common (Woods et al. 2005). MCPH, for example, has been reported in only about 100 families worldwide (Kaindl et al. 2010).

Non-centrosomal genes can also lead to microcephalic primordial dwarfisms, usually accompanied with other malformations. Meier-Gorlin syndrome is characterised by small ears, absent/hypoplastic patellae and short stature (Gorlin et al. 1975). Taybi-Linder syndrome (also known as MOPD type I or III) has profound growth retardation and severe brain malformation of the cerebral cortex (Sigaudy et al. 1998). In patients affected by lissencephaly, the brain is smooth with a thickened cortex, although microcephaly is not always present (Dobyns et al. 1993).

Since the majority of genes found mutated in microcephaly are centrosome or spindle pole-associated genes, we will focus this chapter on these, while mentioning briefly other non-centrosomal genes.

5.4.1.1 Etiology of Microcephaly

So far, three main causes have been proposed to be at the basis of microcephaly. They ultimately converge at the same outcome, depletion of the pool of progenitors during brain development, and consequently fewer cells can form the brain. These three causes are DNA damage response, spindle orientation and spindle integrity (Fig. 5.3).

The first MCPH gene identified, *microcephalin,* has been associated with the DNA damage checkpoint (Jackson et al. 1998). The DNA damage checkpoint maintains cells blocked in G2 through the activation of the G2/M checkpoint, inhibiting entry into mitosis to allow DNA repair. Defects in DNA repair result in apoptosis or premature differentiation, at least in certain cell types (Inomata et al. 2009; Schneider et al. 2013; Sherman et al. 2011).

Defects in spindle orientation have been proposed to result from mutations in at least three MCPH genes, *Aspm*, *CDK5RAP2* (also known as *Cep215*) and *CPAP* (also called *CenpJ*) (Fish et al. 2006; Kitagawa et al. 2011a; Lancaster et al. 2013; Lizarraga et al. 2010). In both symmetric and asymmetric dividing cells, spindle orientation determines the plane of cell division and consequently the correct segregation of cell fate determinants (Morin and Bellaiche 2011). For example, *Aspm* knockdown in the mouse neuroepithelium caused defects in spindle orientation that led to premature differentiation during neurogenesis (Fish et al. 2006).

Certain MCPH mutations cause disruption of centrosome integrity and numerical defects, affecting also spindle formation. Among these, mutations in the master

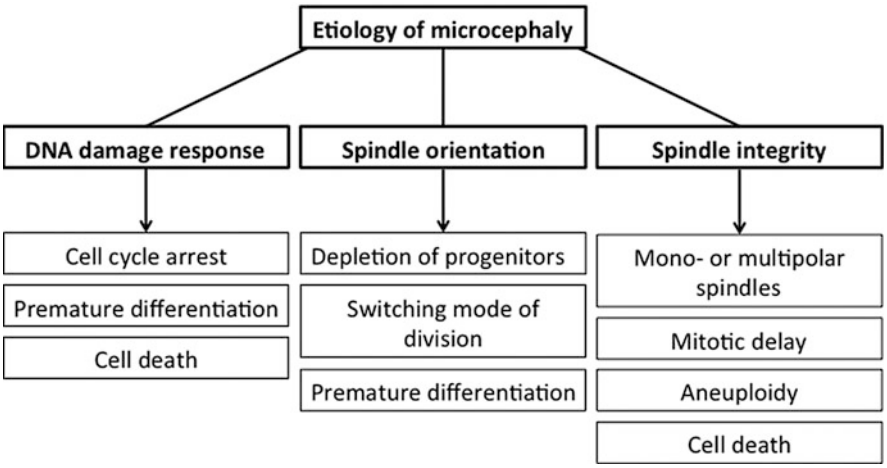

Fig. 5.3 Etiology of microcephaly. Three major causes of microcephaly have been proposed so far: (i) DNA damage response, (ii) spindle orientation, (iii) spindle integrity. Through different means, they all lead to depletion of the pool of progenitors

regulator of centriole duplication *PLK4* have been recently described (Martin et al. 2014; Shaheen et al. 2014). Patient-derived fibroblasts showed reduced protein levels and consequent centriole duplication failure. Mitotic spindle formation was also affected with a predominance of monopolar spindles (Martin et al. 2014).

Another type of centrosome dysfunction that also impacts in brain size is centrosome amplification (Marthiens et al. 2013). Overexpression of *Plk4* in embryonic NSCs resulted in the presence of supernumerary centrosomes in one third of the NSC population. Unexpectedly, failure to cluster led to the generation of multipolar spindles that divided abnormally and generated aneuploid daughter cells. These cells died of apoptosis in a p53-dependent manner, depleting in this way the population of neural progenitors. These results suggest that multipolarity, aneuploidy and consequent cell death can also be at the basis of microcephaly. In agreement, mutations in *STIL*, identified in microcephaly patients, cause centrosome amplification (Arquint and Nigg 2014), and mutations in *Wdr62* cause spindle multipolarity (Chen et al. 2014). In addition, aneuploidy and cell death by apoptosis were also noticed in *asp* (*ASPM* orthologue) *Drosophila* mutants that present defects in head size (Rujano et al. 2013).

Lack of centrosomes or mutations perturbing its integrity frequently result in lengthened mitosis and increased mitotic index as the generation of a bipolar spindle in the absence of centrosomes takes more time (Basto et al. 2006; Bazzi and Anderson 2014; Chen et al. 2014; Insolera et al. 2014; Lizarraga et al. 2010; Sir et al. 2013). Although at the moment a true correlative relationship between mitotic delay and organ size has not been established, it is possible that certain phases of development would require fast proliferation rates at least in certain progenitor

cells. Failure to divide correctly or in a rapid way might induce cell death and also contribute to MCPH (Bazzi and Anderson 2014; Chen et al. 2014; Novorol et al. 2013) (see above).

Interestingly, cell cycle lengthening not related to centrosome dysfunction was proposed to lead to microcephaly. In Maier-Gorlin syndrome, for example, mutations in components of the pre-replicative complex cause defects in the initiation of DNA replication (Bicknell et al. 2011a; Bicknell et al. 2011b; Guernsey et al. 2011). As a consequence, S-phase progression and completion were delayed (Bicknell et al. 2011b). Recently, mutations in kinetochore components, *CASC5* (MCPH4) and *CENP-E* (MCPH13), have also been described in patients with severe microcephalic primordial dwarfism (Genin et al. 2012; Jamieson et al. 1999; Mirzaa et al. 2014). CASC5 is required for correct microtubule attachment to the centromere and the spindle assembly checkpoint (Kiyomitsu et al. 2007), whereas CENP-E is a kinesin required for accurate chromosome congression and segregation (Mirzaa et al. 2014; Putkey et al. 2002; Weaver et al. 2003). Therefore, aneuploidy appears as a possible cause of microcephaly in several size-related syndromes.

5.4.1.2 Genes Identified So Far

Initially when the genetic causes of microcephaly started to be unravelled, it appeared that MCPH and SCKS could be classified not only phenotypically but also genetically (Table 5.1). *CPAP* and *CEP152* were first described as MCPH genes (Bond et al. 2005; Guernsey et al. 2010). Later, both were also identified in families affected by SCKS (Al-Dosari et al. 2010; Kalay et al. 2011). This led to the emerging idea that MCPH and SCKS are not two different diseases but a spectrum of the same disorder with different degrees of penetrance (Verloes et al. 1993). MOPD-II might also be included in this spectrum since *pericentrin* (*PCNT*) was initially reported in families with SCKS (Griffith et al. 2008) and subsequently in patients diagnosed with MOPD-II (Rauch et al. 2008; Willems et al. 2010). For this reason, we will refer to all these syndromes as primordial microcephalic disorders.

To date, twelve centrosome/spindle pole-related genes (Table 5.1) have been identified in primordial microcephalic disorders in humans. Mutations are frequently predicted to result in shorter truncated versions of the affected proteins. Most of our knowledge comes from studies performed in cell lines, and today, we still lack cell or animal models that recapitulate the human mutations as in most cases the few models available are knockdown or knockout approaches that decrease the overall level of wild-type proteins.

Here we describe the known functions of centrosome, spindle pole with mutation described in growth disorders. We will also briefly describe other genes associated with the microtubule cytoskeleton mutated in growth disorders.

Table 5.1 Genes involved in diseases characterised by microcephaly

Gene	Localisation	Disorder	References
MCPH1/ *microcephalin*	Chromatin associated	MCPH	Alderton et al. (2006), Jackson et al. (2002) Jackson et al. (1998)
MCPH2/ *WDR62*	Spindle pole component	MCPH cases with brain malformations	Bilguvar et al. (2010), Chen et al. (2014), Nicholas et al. (2010), Yu et al. (2010)
MCPH3/ *CDK5RAP2* (*Cep215*)	Centrosome (PCM component)	MCPH	Barrera et al. (2010), Bond et al. (2005), Buchman et al. (2010), Lancaster et al. (2013), Lizarraga et al. (2010)
MCPH4/ *CASC5*	Kinetochore	MCPH	Genin et al. (2012), Jamieson et al. (1999)
MCPH5/ *ASPM*	Spindle pole component	MCPH	Bond et al. (2002), Darvish et al. (2010), Pattison et al. (2000), Rujano et al. (2013)
MCPH6/ SCKL4/ *CPAP*	Centriole component	MCPH SCKS	Al-Dosari et al. (2010), Bazzi and Anderson (2014), Bond et al. (2005), Insolera et al. (2014), Kitagawa et al. (2011a)
MCPH7/*STIL*	Centriole component	MCPH	Arquint and Nigg (2014), Kumar et al. (2009), Novorol et al. (2013)
MCPH8/ *CEP135*	Centriole component	MCPH	Hussain et al. (2012)
MCPH9/ SCKL5/ *CEP152*	Centriole and PCM component	MCPH SCKS	Guernsey et al. (2010), Kalay et al. (2011)
MCPH10/ *ZNF335*	Chromatin remodelling protein	MCPH with MCD	Yang et al. (2012)
MCPH11/ *PHC1*	Chromatin remodelling protein	MCPH	Awad et al. (2013)
MCPH12/ *CDK6*	Cytoplasmic and nuclear (interphase), centrosome (mitosis)	MCPH	Hussain et al. (2013)
MCPH13/ *CENP-E*	Kinetochore	Similar to MOPD-II	Mirzaa et al. (2014)
SCKL6/ *CEP63*	Ring around parental centriole	SCKS	Sir et al. (2011)
PLK4	Centriole duplication regulator	SCKS with retinopathy	Martin et al. (2014), Shaheen et al. (2014)
SAS-6	Centriole component	MCPH	Khan et al. (2014)
PCNT	Centrosome (PCM component)	SCKS MOPD-II	Griffith et al. (2008), Rauch et al. (2008)
LIS1	MTs and spindle	LIS	Hattori et al. (1994), Moon et al. (2014), Reiner et al. (1993), Yingling et al. (2008)
DCX	MTs and spindle	LIS	des Portes et al. (1998), Gleeson et al. (1998)

(continued)

Table 5.1 (continued)

Gene	Localisation	Disorder	References
KIF5C	MTs and spindle	Microcephaly with MCD	Poirier et al. (2013)
KIF2A	MTs and spindle	Microcephaly with MCD	Poirier et al. (2013)
DYNC1H1	MTs and spindle	MCD (usually normocephaly)	Poirier et al. (2013)
TUBA1A	MTs and spindle	LIS to MCD	Tischfield et al. (2011)
TUBB2B	MTs and spindle	PMG	Tischfield et al. (2011)
TUBB3	MTs and spindle	MCD	Tischfield et al. (2011)
TUBG1	MTs and spindle	Microcephaly with MCD	Poirier et al. (2013)

The table shows all the genes described so far to be involved in diseases characterised by microcephaly. Genes called MCPH (MCPH 1–13) have been found mutated in patients affected by autosomal recessive primary microcephaly (MCPH). MCPH presents only minor brain malformations. In Seckel syndrome (SCKS) and microcephalic osteodysplastic primordial dwarfism type II (MOPD-II), microcephaly is accompanied with more severe defects such as polymicrogyria, retinopathy or defective neuronal migration. As described in the second column, most of the microcephalic genes encode for centrosomal proteins (centriole structure and PCM) or proteins associated with the mitotic spindle machinery

MCPH autosomal recessive primary microcephaly, *SCKS* Seckel syndrome, *MOPD-II* microcephalic osteodysplastic primordial dwarfism type II, *LIS* lissencephaly, *MCD* malformations of cortical development, *PMG* polymicrogyria

5.4.1.3 Genes Required for Centriole Duplication

1. *CEP152* (known as asterless in flies) is associated with centrioles, and it is required for centriole duplication as it forms a scaffold for the recruitment of PLK4, the master regulator of centriole duplication (Cizmecioglu et al. 2010; Dzhindzhev et al. 2010; Hatch et al. 2010; Kim et al. 2013; Sonnen et al. 2013). In flies it is also required for PCM recruitment (Varmark et al. 2007). *CEP152* mutations were initially identified in patients affected by MCPH (Guernsey et al. 2010) and later also in families with SCKS (Kalay et al. 2011). Mutations are predicted to give loss-of-function truncated proteins. Analysis of fibroblasts and lymphocytes derived from SCKS patient cells showed increased replicative stress and chromosomal instability. In addition, high frequency of abnormal cell divisions with multiple nuclei, fragmented centrosomes and aneuploidy was also noticed (Kalay et al. 2011).
2. *PLK4* is a serine-threonine kinase, member of the polo-like kinase family. Its activity is required for centriole duplication (Bettencourt-Dias et al. 2005; Habedanck et al. 2005). PLK4 self-regulates its own stability through trans-autophosphorylation upon homodimerisation (Guderian et al. 2010; Holland et al. 2010). Two recent studies described mutations in *PLK4* in distinct families (Martin et al. 2014; Shaheen et al. 2014). Individuals displayed profound microcephaly, reduced stature and retinopathy. This latter defect was reported

for the first time in a primordial microcephalic gene (Martin et al. 2014). Patient-derived fibroblasts showed highly reduced PLK4 protein levels and impaired centriole duplication. Although spindle formation was affected, chromosome segregation defects were rarely observed. In a zebra fish model, depletion of *plk4* transcript through morpholino antisense oligonucleotides recapitulated the patients' phenotype: delay in mitotic progression, decreased cell number and consequently also body size reduction. Interestingly, cilia-related phenotypes were seen in a morpholino dose-dependent manner.

3. *CPAP* (known as SAS-4 in flies and worms) is required for microtubule attachment to the initial pro-centriole scaffold, and it controls centriole length and microtubule elongation (Pelletier et al. 2006; Tang et al. 2009). As *CEP152*, mutations in *CPAP* can also lead to both MCPH and SCKS syndromes (Al-Dosari et al. 2010; Bond et al. 2005). A mouse model expressing a truncated variant of CPAP recapitulates many clinical characteristics of SCKS, including intrauterine growth retardation, microcephaly and skeletal defects (McIntyre et al. 2012). DNA damage and apoptosis were also increased in the brain region where cortical neurogenesis takes place and the number of neurons was significantly reduced. A new mouse *Cpap* model, in which *Cpap* was selectively removed from neural progenitors during neurogenesis, also showed a strong microcephalic phenotype (Insolera et al. 2014). Loss of centrioles led to detachment of the neural progenitors from the ventricular zone, where they normally reside. Remarkably, these cells did not change their fate and maintained proliferative capacity. Nevertheless, mitosis was delayed and p53 expression was up-regulated. This led to apoptosis and consequently neuronal loss and microcephaly. Importantly, aneuploidy and DNA damage were not observed (Insolera et al. 2014). The expression of *CPAP* MCPH-mutated versions in human culture cells induced defects in centriole formation and randomised spindle orientation (Kitagawa et al. 2011a). Importantly, one CPAP mutation found in MCPH family impairs centriole formation *in vivo* (Kumar et al. 2009) due to a weaker interaction with STIL (Cottee et al. 2013).
4. Very recently, a mutation in the *HsSAS-6* gene that encodes a protein recruited during the initial steps of procentriole assembly (Kleylein-Sohn et al. 2007; Leidel et al. 2005; Strnad et al. 2007) has been reported in a newly identified MCPH family. This mutation, when expressed in human cells in culture, impaired centrosome duplication, which led to monopolar spindle formation (Khan et al. 2014). So it is possible that in this case abnormal cell division, aneuploidy and consequent cell death of neuronal progenitors contribute to brain size defects.
5. *STIL* (Ana2 and SAS-5 in *Drosophila* and *C. elegans*) is a centriole duplication protein that participates in cartwheel assembly (Arquint et al. 2012; Tang et al. 2011). STIL dissociation from centrosomes during early mitosis triggers HsSAS-6 dissociation and so cartwheel disassembly (Arquint and Nigg 2014). Mutations found in MCPH patients result in the expression of truncated proteins that lack the degradation motif and cause centrosome amplification (Arquint and Nigg 2014; Kumar et al. 2009). Likely, extra centrosomes and consequent

aneuploidy and cell death contribute to MCPH in this case. In addition, since centrosome amplification also causes a delay in mitosis (Basto et al. 2008; Marthiens et al. 2013), it is possible that this also contributes to brain size reduction as discussed above. Interestingly, morpholino-mediated knockdown in zebra fish showed a dramatic increase in both the number of retina progenitors and mitotic cells arrested in prometaphase and increase in apoptotic cells (Novorol et al. 2013).

6. *CEP135* (*Bld10* in flies) has been proposed to act as a bridging molecule between the "cartwheel" and centriole microtubules, being required for CPAP-mediated centriole elongation (Lin et al. 2013). A single mutation has been found so far in one MCPH family. This mutation results in a truncation at the C-terminus (Hussain et al. 2012), which affected the region that mediates CEP135-cartwheel interaction (Lin et al. 2013). Unexpectedly, 22 % of primary fibroblasts derived from patients completely lack centrosomes, while 18 % contained extra centrosomes, or centrosome fragments (Hussain et al. 2012). In this case, it is possible that several types of defects are at the basis of brain size reduction.
7. *CEP63* is an MCPH protein that regulates CEP152 centrosomal localisation to ensure efficient and timely controlled centriole duplication (Brown et al. 2013; Sir et al. 2011). Indeed, human B lymphocytes derived from affected patients showed reduced level of CEP152 at the centrosomes but without major defects in spindle formation and centrosome number (Sir et al. 2011). DT40 chicken B lymphocytes, which have a rapid cell cycle, presented an increased population doubling time and monopolar spindles due to inefficient centriole duplication. It has been proposed that CEP63 is required to timely ensure the presence of enough CEP152 (and consequently PLK4) to allow centriole duplication. Since neural progenitors divide much faster than lymphocytes (10–12 h vs. 24 h), the presence of CEP63 might be essential in the fast proliferating progenitors of the developing brain. Supporting this hypothesis, *Cep63*-deficient mice have neural progenitors with monopolar spindles and acentriolar spindle poles (Marjanovic et al. 2015). As a consequence, these defects delay mitosis, trigger p53-dependent cell death and ultimately lead to microcephaly, similar to the *Cpap*-mutant mouse model (Insolera et al. 2014). Moreover, *Cep63*-deficient mice also showed body growth retardation, recapitulating thus two key characteristics of human SCKS syndrome caused by *CEP63* mutations (Sir et al. 2011). Interestingly, this work also uncovered a surprising function of CEP63 in meiotic male recombination (Marjanovic et al. 2015). The authors proposed that centrosome loss in *Cep63*-deficient spermatocytes impairs normal intranuclear chromosome movement that is required to facilitate homologous chromosomes encounter and thus meiotic DNA recombination, leading to defective spermatogenesis. CEP63 seems to be also a target of the DNA damage response pathway in vertebrate cells. Activation of this pathway promotes CEP63 displacement from spindle poles, inhibiting spindle formation and delaying mitotic progression (Smith et al. 2009). Thus, mutations in CEP63 might perturb cell cycle progression in several ways.

5.4.1.4 Genes Encoding for Centrosomal Proteins

8. *Pericentrin* (PCNT) is a component of the pericentriolar material (PCM), known to play an important role in the recruitment of proteins to the centrosome (e.g. γ-tubulin) (Doxsey et al. 1994; Zimmerman et al. 2004). It was the first centrosomal gene identified in two primordial dwarfism disorders, SCKS and MOPDII (Griffith et al. 2008; Rauch et al. 2008). In lymphoblastoid SCKS patient cell lines defective ATR signalling pathway and lack of G2-M checkpoint after UV radiation were also reported (Griffith et al. 2008). Importantly however, monopolar spindles with decreased γ-tubulin recruitment were also noticed, raising the possibility that these abnormal spindles also contribute to abnormal chromosome segregation and aneuploidy in cells with PCNT mutations.
9. *CDK5RAP2* (Cep215 and Cnn) is a PCM protein involved in γ-tubulin recruitment (Fong et al. 2008). CDK5RAP2 seems to be involved in centriole engagement and maintenance of the neural progenitor pool in the mouse developing neocortex (Barrera et al. 2010; Buchman et al. 2010). Embryonic fibroblasts derived from mouse models carrying *Cdk5rap2* mutations similar to the ones found in human MCPH showed centrosome amplification due to loss of centriole engagement and consequent formation of multipolar spindles (Barrera et al. 2010). *Cdk5rap2* knockdown by *in utero* electroporation described a depletion of neural progenitors in the developing mouse neocortex due to premature neural differentiation (Buchman et al. 2010). However, in these two studies, neither spindle orientation nor microcephaly was observed. Importantly, an *in vitro* model of human brain development that used reprogrammed skin fibroblasts from MCPH patients showed reduced neuroepithelial tissue with defects in spindle orientation and premature neural differentiation (Lancaster et al. 2013). Spindle positioning might, however, not be the sole defect as the characterisation of Hertwig's anaemia mouse model, which carries a mutation in the *Cdk5rap2* gene, also showed multipolar spindles in neural progenitors accompanied by cell death (Lizarraga et al. 2010).
10. *CDK6*, in concert with CDK4, regulates the G1/S transition (Meyerson and Harlow 1994). It localises in the cytoplasm and in the nucleus in interphase and also at the centrosome throughout mitosis (Hussain et al. 2013). Fibroblasts from MCPH patients do not contain centrosomal CDK6 during mitosis. This results in several defects such as disorganised interphase microtubule network and mitotic spindles, centrosome amplification, reduced proliferation and cell death (Hussain et al. 2013). Although *Cdk6*-null mice do not show microcephaly at birth, CDK6 is required during adult neurogenesis. Lack of this kinase resulted in lengthened G1 and consequent premature cell cycle exit (Beukelaers et al. 2011; Malumbres et al. 2004). Absence of microcephaly in *Cdk6*-null mice suggests that the particular mutation found in MCPH patients might have a more severe effect in brain development than loss of CDK6.

5.4.1.5 Genes Encoding for Spindle Pole-Associated Proteins

11. The abnormal spindle-like microcephaly-associated (*ASPM*) gene is the most frequently mutated locus found in MCPH (Bond et al. 2002; Darvish et al. 2010; Pattison et al. 2000). ASPM is a microtubule minus end- and spindle pole-associated protein with important roles in cell division (do Carmo Avides and Glover 1999; Gonzalez et al. 1988; Gonzalez et al. 1990; Riparbelli et al. 2002; Saunders et al. 1997; Wakefield et al. 2001). Loss of ASPM causes alteration in spindle positioning in mouse neural stem cells, which favours asymmetric cell division depleting the pool of progenitors (Fish et al. 2006). Recent work in *Drosophila* showed that the ASPM orthologue, Asp, also plays a role in brain size regulation in flies (Rujano et al. 2013). Defects in spindle orientation, chromosome segregation and interkinetic nuclear migration were noticed. Moreover, Asp was found to interact with myosin II, and this interaction was essential during brain morphogenesis to maintain neuroepithelial organisation (Rujano et al. 2013). These results showed that Asp plays unexpected functions, beyond the role in microtubule cytoskeleton in brain development. They might also explain the observations that some MCPH mutations also affect brain organisation in addition to size (Mochida 2005).
12. *WDR62* is the second most common mutated gene in MCPH. It is a spindle pole protein-coding gene (Bilguvar et al. 2010; Nicholas et al. 2010; Yu et al. 2010). It has been recently demonstrated in a hypomorphic *Wdr62* mouse model that neural progenitor cells are arrested in mitosis due to spindle stability defects with increased cell death. In addition, defects in spindle positioning or premature differentiation were not seen, suggesting that disruption of mitotic progression and consequent cell death of neural progenitors is a potential cause of human microcephaly (Chen et al. 2014).

5.4.1.6 Genes Encoding for Molecular Motors and Microtubule-Associated Proteins

Defects in molecular motors and microtubule-associated proteins lead to severe disorders with microcephaly and brain malformation. Lissencephaly is characterised by the absence of normal folds in the cerebral cortex due to defective neuronal migration (Dobyns et al. 1993). The first gene identified in lissencephaly was *LIS1* (Hattori et al. 1994; Reiner et al. 1993), which encodes a subunit of the cytoplasmic dynein complex. An *in vivo* study demonstrated its requirement for neuronal migration (Reiner et al. 1995). Interestingly, LIS1 has also been implicated in spindle positioning of apical neural progenitors in mouse (Yingling et al. 2008), and recently centrosome amplification and severe chromosome segregation defects have also been described in *Lis1* mutant MEFs (Moon et al. 2014), suggesting that aneuploidy and cell death might also contribute to the overall phenotype. Neuronal migration is impaired in mutation in *Doublecortin* (*DCX*),

and patients carrying this mutation also present lissencephaly (des Portes et al. 1998; Gleeson et al. 1998).

Mutations in *KIF5C* and *KIF2A*, members of the kinesin superfamily, and in *DYNC1H1*, cytoplasmic dynein 1 heavy chain 1, have also been recently described in patients affected by lissencephaly with microcephaly (Poirier et al. 2013). These mutations affect ATP hydrolysis, protein folding and microtubule binding.

Mutation in another motor, *KIF11* (kinesin Eg5), was found to lead to syndromes characterised by microcephaly accompanied by eye malformations (Ostergaard et al. 2012). Eg5 is a mitotic kinesin involved in centrosome separation (Kwok et al. 2004) and centrosome clustering (Drosopoulos et al. 2014). These results suggest that neural progenitors are more vulnerable to microtubule mutations than other cell types during embryonic development. A further demonstration of the importance of functional microtubules was given by the fact that mutations in *α*-, *β*- and *γ-tubulin* isotypes coding genes also lead to microcephaly with brain malformations (Poirier et al. 2013; Tischfield et al. 2011). Importantly, all human mutations identified in these genes are heterozygous missense mutations.

For further discussion of neurodevelopmental defects caused by an impaired microtubule cytoskeleton, please also see the Chap. 4 by Sánchez-Huertas, Freixo and Lüders.

5.4.1.7 Genes Encoding for Chromatin Associated Proteins

Microcephalin (MCPH1) was the first mutated locus identified in patients affected by MCPH (Jackson et al. 1998). Microcephalin is highly expressed in the developing mouse forebrain, in particular in the region where neural progenitors reside (Jackson et al. 2002). It localises to the DNA during interphase, and it has a role in chromosome condensation. Furthermore, microcephalin mediates the DNA damage response, being recruited to the damaged foci (Lin et al. 2005; Rai et al. 2006; Xu et al. 2004). MCPH1 also localises at centrosomes in U2OS cells (Zhong et al. 2006), in chicken DT-40 cells after irradiation (Jeffers et al. 2008), and recruits Chk1, a kinase involved in the G2-M checkpoint (Alderton et al. 2006). Importantly, however, human lymphoblastoid cell lines with truncating mutations found in MCPH patients do not show impaired DNA damage response, but rather a defective G2-M checkpoint. In these cells, Chk1 is not targeted to the centrosome and mitosis starts even in the presence of damaged DNA, leading to nuclear fragmentation and centrosome amplification (Alderton et al. 2006). Studies from *Drosophila* suggested a role for MCPH1 (also known as *awol*) in chromosome condensation but not in the DNA damage response (Brunk et al. 2007; Rickmyre et al. 2007). A centrosomal localisation of MCPH1 during mitosis has been reported in *Drosophila* embryos (Brunk et al. 2007). However, different to all the other centrosomal/spindle pole MCPH genes, a clear spindle function has not been identified.

In addition to cytoskeleton genes, mutations in the nuclear zinc finger 335 (*ZNF335*, trithorax group) and *PHC1* (polycomb group) genes have been

recently identified in families with severe microcephaly, suggesting a broader cause resulting from alterations in gene expression (Awad et al. 2013; Yang et al. 2012).

5.4.2 Centrosome Defects and Cancer

Centrosome defects and in particular centrosome amplification are usually linked to cancer. Centrosome amplification is present in almost all solid and haematological tumours described (Chan 2011) (Fig. 5.4). At the beginning of the twentieth century, German zoologist Theodor Boveri proposed that centrosome amplification and consequent aneuploidy could be at the basis of tumour initiation (Boveri 2008). This hypothesis was proposed after the observations that the presence of extra centrosomes in sea urchin embryos, due to dispermic fertilisation, could lead to abnormal mitosis and defects in chromosome segregation. These defective chromosome combinations were usually detrimental for embryo development, but Boveri could observe rare cases where abnormal cells continued to proliferate. Boveri remarkably found that they were similar to tumour cells (Boveri 2008). At his time it was already known that aneuploidy was a characteristic of human tumours. Indeed, in 1890 David Hansemann initially observed asymmetric chromosome segregation in human epithelial cells, and he documented this phenomenon in a variety of tumours (Boveri 2008).

Persistent high levels of chromosome mis-segregation, commonly referred to as "chromosomal instability" (CIN), are hallmark of most cancers (Lengauer et al. 1997). It is difficult to understand how centrosome amplification contributes to CIN, since multipolarity is often associated with poor viability. A link between centrosome amplification and viable CIN has been established recently. The transition from multipolarity to bipolarity during the process of clustering promotes merotelic attachments that might lead to viable aneuploid daughter cells (Ganem

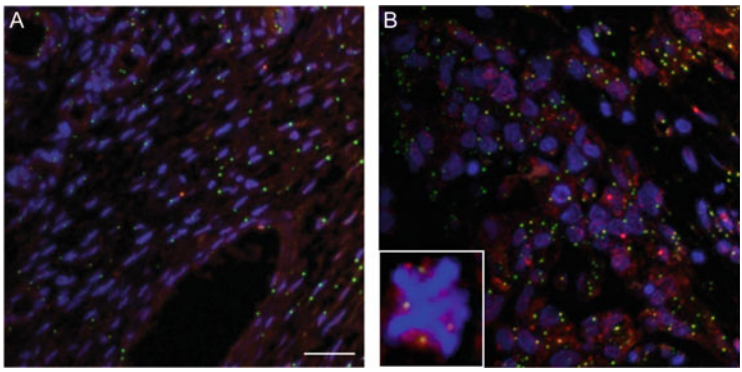

Fig. 5.4 Centrosome amplification in human ovarian tumour. Human ovarian tissue (**a**) and serous tumour (**b**) sections immunostained for pericentrin (*green*), γ-tubulin (*red*). DNA is shown in *blue*. Scale bar = 30 μm

et al. 2009; Silkworth et al. 2009). The contribution of aneuploidy (generated independently of centrosome amplification) to tumorigenesis is tissue dependent. While aneuploidy can promote tumour formation in certain tissues upon carcinogenic treatment, it can also inhibit tumorigenesis in other tissues (Silk et al. 2013; Sotillo et al. 2007; Weaver et al. 2007).

Centrosome amplification was shown to initiate tumorigenesis in *Drosophila* both independently and dependently of aneuploidy. Allograft transplantation of larval brains carrying extra centrosomes caused over-proliferation and tumours. Although defects in chromosome segregation were not observed, defects in mitotic spindle positioning resulted in the increase of the neural stem cell pool (Basto et al. 2008). In flies, mutations that perturb neural stem cell asymmetric cell division due to centriole duplication defects or mutations in polarity genes are tumorigenic with little if any CIN (Castellanos et al. 2008; Caussinus and Gonzalez 2005). In another tissue, the wing imaginal disc, extra-centrosomes, are not efficiently clustered or inactivated with consequent multipolar spindle formation. This generates aneuploid cells and causes tumours in allograft transplantation (Sabino et al. 2015).

In vertebrates the contribution of centrosome amplification to tumorigenesis is still an open question. Overexpression of *Plk4*, which still remains the most efficient mean to drive centriole over-duplication *in vivo*, in the mouse developing central nervous system resulted in microcephaly (Fig. 5.2), but brain tumours were not reported (Marthiens et al. 2013). Although centrosome clustering allows the assembly of bipolar spindles in most embryonic neural stem cells, in a significant proportion of cells, mainly during early and mid-neurogenesis, tripolar spindles and abnormal chromosome segregation lead to the generation of unviable aneuploid cells. These cells died by apoptosis in a p53-dependent manner. Importantly, even in the absence of p53, tumours were not detected in the CNS. It is therefore possible that during development, centrosome amplification and aneuploidy are not sufficient to initiate tumour formation. It will be important in the future to establish whether centrosome amplification during adult life in the mammalian brain or in highly proliferative tissues such as the intestine or the skin is able to drive tumour formation.

Until recently, centrosome amplification was thought to only contribute to tumour formation through the generation of aneuploidy and spindle positioning defects. However, it is also possible that the presence of extra centrosomes even in interphase cells might represent an advantageous condition. Surprisingly, non-transformed human mammary epithelial cells with extra centrosomes showed increased microtubule nucleation capacity that strongly correlated with invasive behaviour (Godinho et al. 2014). Increased centrosomal microtubule nucleation during interphase activates the small GTPase Rac1, which is known to promote invasiveness and metastasis (Mack et al. 2011). It is therefore possible that centrosome amplification contributes to tumour formation in several different ways.

5.5 Conclusions

At the beginning of the last century, it was proposed that defects in centrosome number, in particular centrosome amplification, might be a cause for tumour formation (Boveri 2008). Today, several lines of evidence support this view (Basto et al. 2008; Castellanos et al. 2008; Ganem et al. 2009; Godinho et al. 2014; Nigg 2006; Sabino et al. 2015; Zyss and Gergely 2009). However, the observations made during the past 15 years using autozygosity mapping techniques and whole-genome SNP genotyping implicated centrosome mutations in growth defective syndromes, but not in cancer.

Both primordial dwarfisms and MCPH are characterised by proportionate reduction of body or head size, which results from premature depletion of progenitors and/or increased levels of cell death. In most tumours, cancer cells show high levels of proliferation, and even if high cell death rates can be identified, proliferation and capacity to evade cell death signals are essential during cancer progression and invasion (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011). It is important to mention that most of the cellular pathways affected in MCPH or in primordial dwarfisms are also frequently referred to be dysfunctional in cancer cells. This is the case for DNA damage response (Lord and Ashworth 2012), spindle orientation (Gonzalez 2007) or abnormal cell division and aneuploidy (Boveri 2008). It is therefore possible that these conditions just represent two sides of the same coin. Centrosome dysfunction due to zygotic mutations, if viable, would lead to growth defects such as MCPH or dwarfism. If acquired in somatic adult tissues in certain contexts, they might lead to the opposite effect: over-proliferation and growth. Interestingly, mutation in *BUBR1* and *CEP57* (kinetochore and centrosomal proteins, respectively) leads to a disease called mosaic variegated aneuploidy (MVA), which is characterised by the appearance of tumours at early age and features of primordial dwarfism (microcephaly and short stature) (Hanks et al. 2006; Snape et al. 2011).

Further work is required to understand the relation between centrosome mutations with cancer, MCPH and primordial dwarfism. Can centrosome amplification or any other types of centrosome dysfunction initiate tumorigenesis in humans? And if yes, by which means? Which adult tissues are more prone to develop cancer when accumulating centrosome defects? And concerning growth defects, why is the brain the most susceptible tissue to centrosome mutations? For all these reasons, the centrosome field remains an active one and calls for *in vivo* investigations that will keep us busy in the years to come.

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Microtubules Regulate Cell Migration and Neuronal Pathfinding

6

Ulrike Theisen and Anne Straube

Abstract

While many cell types are able to generate cellular movement through the action of the actomyosin cytoskeleton alone, microtubules are important for establishing and maintaining polarity, regulating the force-generating machinery and cell adhesion. Therefore, directionally persistent cell migration and neuronal pathfinding often require microtubules.

The microtubule cytoskeleton itself is organised asymmetrically to allow differential regulation of the migration machinery at the front and the rear of the cell. Microtubules position organelles such as the nucleus, the centrosome and the Golgi. Transport of mRNAs, vesicles, receptors and signalling components to the cell edges occurs along microtubules. These cargoes in turn support force generation by the actin cytoskeleton, act as a source of membrane lipids and regulate polarity signalling, adhesion, cell-cell communication and chemical gradient sensing. Microtubules themselves and especially the dynamic plus ends act as signalling platforms to control adhesion turnover and membrane protrusion. The rapid turnover of microtubules allows cells to quickly adapt to extracellular signals and change migration direction in response to guidance cues. Microtubule dynamics and organisation are in turn controlled by cortical cues. These feedback mechanisms ensure robustness and adaptation to environmental influences.

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Given the fundamental importance of cell migration for embryonic development, the immune system and wound healing, impaired microtubule function leads to birth defects and diseases. Likewise, drugs targeting microtubules are routinely used to prevent excessive cell migration in cancer metastasis and chronic inflammatory diseases.

6.1 Introduction

Cell migration is a fundamental biological phenomenon occurring in protists as well as in multicellular organisms. Locomotion of unicellular organisms enables access to nutrients and optimal environmental conditions as well as the assembly of cells into spore-bearing structures (Van Haastert and Devreotes 2004). In multicellular organisms, migration is essential to positioning each cell in the body at its correct location. During embryonic development, many cells are generated from precursors in a different location to where they are needed. Furthermore, neuronal precursors in mammals need to migrate not only to reach a specific destination but also to encounter the correct type of cells along the way to form contacts with in order to build the neuronal network in the brain. For example, cerebellar granule cell precursors migrate tangentially until they change to radial migration along glial fibres during which they establish contacts with Purkinje cells needed for the proper wiring of the adult cerebellum (Cooper 2013; Komuro and Rakic 1998; Fig. 6.1a). Other instances of migration occurring during development are clusters of cells that move along the entire length of the body to form the lateral line organ in fish (Fig. 6.1b) and precursors of muscle cells that align before fusion into muscle fibres (Revenu et al. 2014; Wakelam 1985). In adults, cell migration is of utmost importance for immune surveillance and response and for healing wounds (Fig. 6.1c). Finally, defective regulation of migration contributes to chronic inflammatory diseases such as gout and atherosclerosis and enables the spreading of cancer cells from the primary tumour site (Chi and Melendez 2007; Colvin et al. 2010; Friedl and Wolf 2003, Fig. 6.1d). Metastasis is responsible for 90 % of cancer deaths (Mehlen and Puisieux 2006). For an overview of human diseases directly linked to defective migration as a result of an impaired microtubule cytoskeleton, please refer to Table 6.1.

Cells can migrate in various different modes that depend on the environment they are in and on the cell type. On a flat surface such as a plastic dish in culture or the surface of a muscle fibre or endothelial sheets in vivo, cells move in a mesenchymal mode with adhesion to the surface being a crucial aspect of migration. Moving through dense 3D matrices or other confined spaces requires only little adhesion as under these conditions contractile forces that drive amoeboid or blebbing motion can generate forces and traction at the same time. Cells can migrate as individual cells or as collectives, and they can also switch between different types of migration (Friedl and Gilmour 2009). Such a change occurs, for example, during epithelial-mesenchymal transition, a process where cancer cells undergo dedifferentiation from a tissue collective to a more single-cell-like behaviour and acquire the ability to metastasise (Friedl and Wolf 2003).

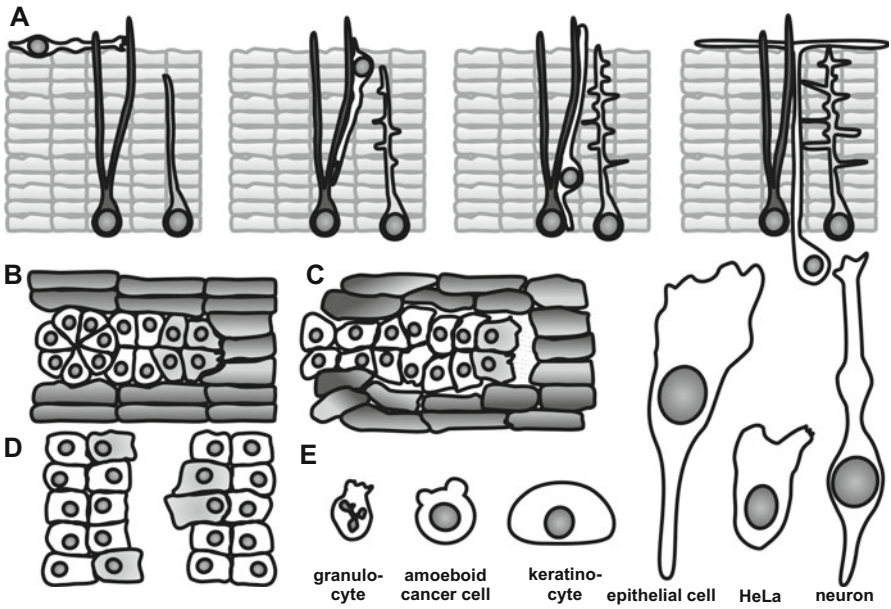

Fig. 6.1 Examples of migration modes and cell shapes. (**a**) Modes of migration depend on the cellular environment. In this example, a cerebellar granule cell neuron (*white*) is migrating tangentially over other tissue, until it finds a glia cell (*dark grey*). It is then guided along the axon of the glia cell during radial migration using microtubule-dependent nucleokinesis to reach the inner layer of the developing cerebellum. During radial migration, the axon of the granule cell projects from the rear of the cell and establishes contacts to Purkinje cells (*light grey*) (Fahrion et al. 2012). (**b**) Some cells migrate as highly coordinated multicellular strands during development. In this example, leader cells form a path for the follower cells. In the lateral line primordium, these take the form of rosettes. Close communication between leader and follower cells is necessary to achieve collective migration (Revenu et al. 2014). (**c**) Similarly, cancer cells often metastasize as clusters of cells that force their way by localised release of extracellular matrix-degrading enzymes (Friedl and Gilmour 2009). (**d**) Wound healing can be recreated in cell culture. Typically, once a gap is created, individual cells (leader cells, *light grey*) at the edge of the wound sense the gap and respond by extending a lamellipodium into the gap. Once these cells start to invade the open space, they are followed by other cells pushing from behind (Tsai et al. 2014). (**e**) Commonly observed migrating cell types are depicted in their relative size. All cells migrate towards the top of the page. Note that the cell area depends on the mode of migration and the stiffness of the substrate and can therefore change depending on the cellular environment

In general, cells need to coordinate the following steps in order to achieve migration (Etienne-Manneville 2013; Ridley et al. 2003):

1. Protrusion. This involves the cell membrane to be pushed forward by cytoskeletal polymerisation.
2. Adhesion. Forces that the cytoskeleton generates must be transmitted to the underlying substratum while regulating the turnover (lifetime) according to the spatial cues (strong attachment at the front, weakening attachment at the rear).

Table 6.1 Diseases directly associated with cell migration and microtubule function

Cause/ accelerating factor	Effect	Disease	Reference
Microtubule structure and stability:			
Dynamic microtubules	Increased motility	Metastasis	Mehlen and Puisieux (2006)
Dynamic microtubules	Immune cell migration	Rheumatoid arthritis	Brahn et al. (1994), Friedl and Weigelin (2008)
Dynamic microtubules	Neutrophil migration	Gout	Chia et al. (2008)
Dynamic microtubules	Infiltration of immune cells into the brain; multiple sclerosis-like phenotype	Experimental autoimmune encephalomyelitis	O'Sullivan et al. (2013)
TUBB3	Neuronal migration defects	Malformation of cortical development	Poirier et al. (2010), Saillour et al. (2014)
TUBA1A	Neuronal migration defects	Lissencephaly/ pachygyria	Poirier et al. (2007)
TUBB2B	Neuronal migration defects	Polymicrogyria	Jaglin et al. (2009)
TUBB5	Neuronal migration defects	Microcephaly	Breuss et al. (2012)
TUBA3A	Neuronal migration defects	Polymicrogyria	Keays et al. (2007)
TUBG1	Neuronal migration defects	Malformation of cortical development	Poirier et al. (2013)
Doublecortin	Neuronal migration defects	Lissencephaly	Gleeson et al. (1999b), Pilz et al. (1998)
MAP1B	Neuronal migration defects	Diverse neuropathologies	Del Rio et al. (2004), Riederer (2007)
APC	Impaired neuronal network formation	Schizophrenia, autism	Cui et al. (2005), Kozlovsky et al. (2002), Mohn et al. (2014)
HDAC6	Blood vessel formation	Tumour angiogenesis, metastasis	Li et al. (2011), Wu et al. (2010)
Clip-170	Increased vessel density in tumours	Tumour angiogenesis	Sun et al. (2013)
Microtubule length and array control:			
Tau	Increased microtubule severing	Alzheimer's disease	Sapir et al. (2012)

(continued)

Table 6.1 (continued)

Cause/ accelerating factor	Effect	Disease	Reference
Katanin	Sperm motility defective	Male fertility defect	O'Donnell et al. (2012)
Spastin	Impaired microtubule severing	Metastasis	Draberova et al. (2011)
Kif2A	Enhanced cell motility and invasiveness	Metastasis	Wang et al. (2010), (2014)
Motor proteins and their regulation:			
Dynein	Movement of organelles, retrograde trafficking affected	Charcot-Marie-Tooth disease type 2; several neurological symptoms	Willemsen et al. (2012)
Lis1	Impaired dynein function	Lissencephaly, Miller-Dieker syndrome	Pilz et al. (1998), Badano et al. (2005), Hattori et al. (1994)
Kif5C	Neuronal migration defects	Malformation of cortical development	Poirier et al. (2013)
Centrosome:			
DISC1	Centrosomal function impaired	Schizophrenia, depression, bipolar disorder	Duan et al. (2007), Hashimoto et al. (2006), Hennah et al. (2009), Ishizuka et al. (2011), Meyer and Morris (2009), Steinecke et al. (2012)
PCM1	Centrosomal satellites defective	Schizophrenia	Kamiya et al. (2008)
SDCCAG8	Centrosomal satellites defective	Schizophrenia	Hamshere et al. (2013), Insolera et al. (2014)
BBS1, BBS4	Defective cilia; defects in migration cause craniofacial dysmorphia	Bardet-Biedl syndrome	Tobin et al. (2008)
Excess centrosomes in interphase	Impaired migration	Angiogenesis; defective vessel sprouting	Kushner et al. (2014)
Cell polarity signalling:			
Cdc42	Stability of microtubules in the uropod of neutrophils	Immunodeficiency	Kumar et al. (2012)
Cell adhesion:			

(continued)

Table 6.1 (continued)

Cause/ accelerating factor	Effect	Disease	Reference
ACF7	Microtubule-regulated adhesion turnover defective	Delayed skin healing	Wu et al. (2008)
APC	Cell adhesion by cadherins affected	Tumour development/ metastasis in colorectal cancer	Faux et al. (2004)

3. Contraction. Actin and myosin generate contractile forces to move the cell body forward.
4. Retraction. Substrate adhesion at the rear must be released and the rear end of the cell brought forward.

This classic model describes the series of events needed to propel a cell forward. The importance of each of these aspects differs depending on the type of migration, e.g. mesenchymal migration strongly depends on attachment, while amoeboid migration does not (for details, see Lammermann and Sixt 2009). In order to achieve persistent directional motility of a cell, there are essential requirements that need to be met: First, cell polarity needs to be established. Next, the cytoskeleton needs to be arranged so that forces are generated in the different parts of the cell that allow protrusion at the front and retraction at the rear. These forces need to be transferred to the underlying substrate with the help of adhesive contacts, either to the extracellular matrix (ECM) or to neighbouring cells. Additional tasks are added where cells migrate in clusters, as contacts and communication between the migrating cells need to be maintained at all times.

Although migration is often regarded as a purely actin-driven process, microtubules have fundamental roles in the regulation of different aspects of the complex task of moving a cell forward. However, the exact involvement of microtubules in migration is strongly dependent on the type of cell and its environment. Leaving aside protists, whose motility depends entirely on microtubules organised into cilia, it appears that in small cells, such as neutrophils (Dziezanowski et al. 1980; Niggli 2003), T cells (Takesono et al. 2010) or fish keratinocytes (Euteneuer and Schliwa 1984), microtubules are dispensable for efficient migration, even if some aspects of migration require microtubules (Stramer et al. 2010; Vogl et al. 2004, Fig. 6.1e). This was demonstrated in experiments using microtubule-depolymerising drugs, e.g. nocodazole or colcemid. When small cell types were treated with these drugs, their migration was hardly impaired or even stimulated (Euteneuer and Schliwa 1984; Niggli 2003). Yet when the experiment was repeated on larger cell types, such as fibroblasts, neurons, astrocytes or cancer cells, the effects on migration ranged from loss of directionality and cell polarity and

reduction of speed to complete inhibition of cell locomotion (Etienne-Manneville 2004; Ganguly et al. 2012; Liao et al. 1995; Vasiliev et al. 1970; Xu et al. 2005). One idea is that diffusion or actin-based transport can efficiently compensate for loss of microtubules in small but not in larger cells (Kaverina and Straube 2011; Keren et al. 2008).

By their reach throughout the whole cell, microtubules can coordinate the different aspects involved in cell migration by acting at different parts of the cell at the same time (Fig. 6.2), such as regulating increased adhesiveness at the cell front while reducing adhesiveness at the rear. They are also crucial to long-distance transport and directing cargo (vesicles, proteins, mRNA) to different regions of the cell, thereby gaining a regulatory influence over local protrusion and adhesiveness, signal perception/transduction and cell-cell communication. In addition, their mechanical properties contribute to shaping the cell, for example, by preventing the collapse of membrane structures due to their resistance to compression. Still, the microtubule system is fairly short-lived, as a result of the intrinsic dynamic instability, allowing the microtubule cytoskeleton to adapt very quickly to changes, for example, when signals from the environment are perceived that make changes to the migration direction necessary. In spite of their normally short lifetime, certain microtubules can be stabilised for specific functions, e.g. in order to move the nucleus forward during neuronal migration. Finally, by selectively adapting the composition of proteins binding to the dynamic plus ends, these can provide a spatially and temporally highly restricted environment to carry out special tasks, such as targeting focal adhesions at the rear of the cell for disassembly or interacting with signalling components in a very controlled manner.

6.2 Microtubule Organisation in Migrating Cells

In many migrating cells, microtubules show an asymmetric arrangement. This is typically biased towards the front of the cell in most cell types, such as fibroblasts, epithelial and endothelial cells, astrocytes and neurons (Fig. 6.2a, b), but a bias to the rear of the cell has been shown in leucocytes (Kaverina and Straube 2011; Watanabe et al. 2004; Yoo et al. 2012). Many microtubules are nucleated by and anchored with their minus ends at the centrosome so that their dynamic plus ends project towards the cell cortex. Often the centrosome is positioned between the nucleus and the leading edge of the cell. The mechanism behind orienting the centrosome involves microtubule capture at the cortex, which allows the minus-end-directed motor dynein to exert pulling forces on the microtubules to position the centrosome in the cell centre (Palazzo et al. 2001; Tsai and Gleeson 2005; Yvon et al. 2002; Fig. 6.3). In addition, actin-mediated forces pull the nucleus backwards (Gomes et al. 2005). While defects in the positioning of the centrosome are indicative of problems in cell polarity and correlate with cell migration defects (Etienne-Manneville and Hall 2003; Luxton and Gundersen 2011; Tsai et al. 2007), it is unlikely that the position of the centrosome itself determines directionality of cell migration or the asymmetry of the microtubule network. Centrosome position

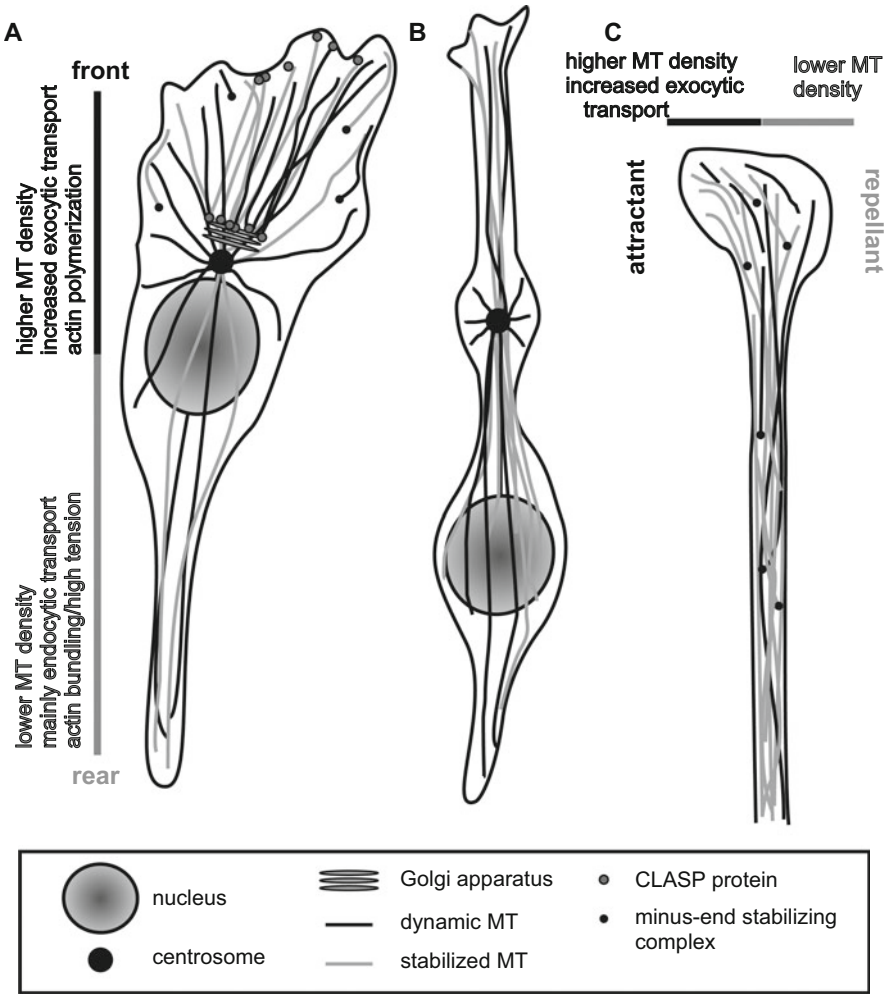

Fig. 6.2 Microtubule arrangement in migrating/protruding cells. (**a**) In epithelial cells moving over a flat surface, more microtubules reach the leading edge than the cell rear. The centrosome nucleates a radial array of microtubules, but rearwards growing microtubules are deflected by the nucleus. In addition, the trans-Golgi nucleates a front-directed microtubule array. This front bias is enhanced by a gradient of microtubule-destabilising factors, which are more active at the rear of the cell and the selective stabilisation of microtubules at the leading edge mediated by plus end capture at the cell cortex. CLASP proteins have been implicated in both nucleation at the Golgi and capture at the cell cortex. (**b**) In migrating neurons, most microtubules extend towards the leading edge, and only few reach around the nucleus to the rear. Microtubules are nucleated from the centrosome, which is oriented towards the leading edge. A cage of stable microtubules links the centrosome and the nucleus. This cage is important for moving the nucleus forward. (**c**) Growing axons resemble migrating cells in many aspects. They typically exhibit a dense array of stable microtubules. Microtubules nucleated at the centrosome are often not long enough to reach the leading edge. Instead the array mainly contains free microtubules generated by microtubule severing and capping of the minus ends by stabilising complexes. These microtubules can be moved by motor proteins and contribute to force generation

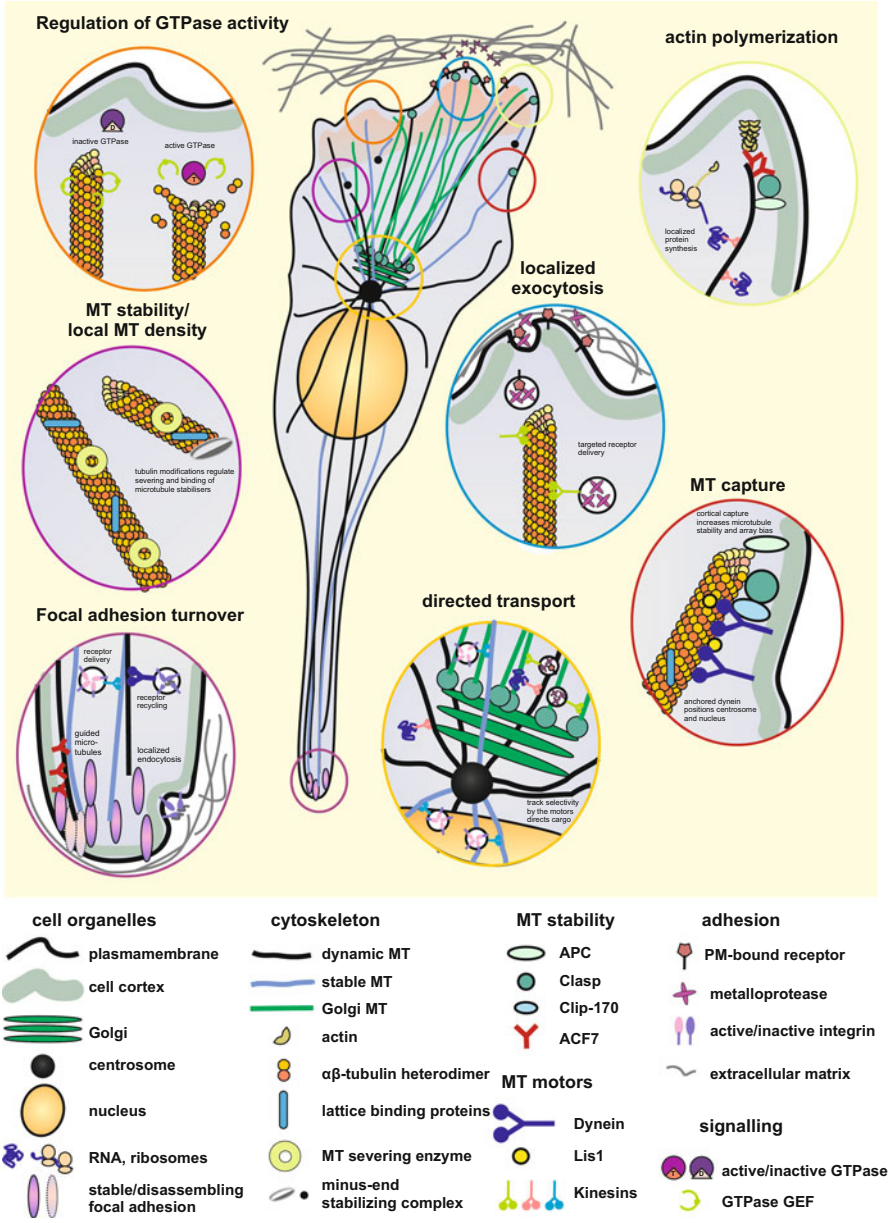

Fig. 6.3 Microtubule functions in cell migration. Microtubules are the main tracks for intracellular long-distance transport, delivering cargo to support and regulate the cell migration machinery. Microtubules are organised asymmetrically and their stability is regulated spatially by rescue factors such as CLASPs, minus end capping and severing proteins. Dynamic microtubules modulate Rho GTPase signalling by sequestering, concentrating and releasing regulatory proteins. Microtubules stimulate actin polymerisation through delivery of mRNAs and the accumulation of actin nucleators such as APC and formins at microtubule ends. Localised exocytosis supplies the membrane for protrusion and receptors and enzymes for matrix degradation. Microtubule targeting and directed transport also regulates focal adhesions

is dictated by cell geometry and therefore a read-out of cell shape (Dupin et al. 2009; Gomes et al. 2005). Most cells moving on 2D surfaces will position the centrosome in front of the nucleus. However, plating the same cells on patterned substrates that confine adhesion to narrow lines results in an elongated cell morphology and efficient cell migration, but the centrosome is found behind the nucleus (Pouthas et al. 2008). Under these conditions, the majority of microtubules still grow towards the front of the cells; therefore, centrosome position and microtubule network bias are independent of each other (Straube, unpublished data). Similarly, in wound-edge Ptk cells, the rear-oriented position of the centrosome may become compensated for by actin-based transport of microtubules to the front (Yvon et al. 2002). Likewise, zebrafish neutrophils migrating in vivo position the centrosome in front of the nucleus, but the majority of microtubules project towards the rear (Yoo et al. 2012) (for an overview of centrosomal positions in different systems, see Luxton and Gundersen 2011). The mechanisms underlying the reverse microtubule orientation in these cells are not understood, and we will concentrate on the more commonly observed front-biased microtubule organisation in the remainder of this chapter.

The Golgi complex is usually positioned close to the centrosome (Kupfer et al. 1983; Pouthas et al. 2008) and nucleates a large number of almost exclusively front-directed microtubules from the trans-Golgi network (Chabin-Brion et al. 2001; Efimov et al. 2007; Rivero et al. 2009; Fig. 6.3). As the centrosome and the associated microtubules organise the Golgi apparatus, this coupling of centrosomal positioning and Golgi-mediated nucleation of microtubules increases the front-biased orientation of microtubules in the cell (Vinogradova et al. 2012). An extreme example of higher microtubule density extending towards the front occurs in the very long, but narrow, lamellipodia of migrating granule cell neurons (Umeshima et al. 2007).

Only a few of all microtubules growing towards the leading edge of the cell actually reach the plasma membrane. These are so-called "pioneer" microtubules (Etienne-Manneville 2013). Most other front-oriented microtubules terminate near the actin-rich regions of the cortex, but do not touch the expanding membrane at the front. It is thought that retrograde flow from the actin filaments prevents these microtubules from reaching the membrane (Waterman-Storer and Salmon 1997). "Pioneer" microtubules withstand expulsion by actin retrograde flow by anchorage to the membrane (Etienne-Manneville et al. 2005). The observation that "pioneer" microtubules show extensive tubulin modifications supports the idea of increased longevity of this microtubule population (Bulinski and Gundersen 1991; Gundersen and Bulinski 1988). A similar arrangement is found in axons, where only a subset of microtubules enters the peripheral domain of the growth cones (Fig. 6.2c).

In differentiating neurons, the cell body will no longer move forward, but the growth cones at the tips of the extending neurites structurally and functionally resemble the lamella of migrating cells. Growth cones are able to continue to grow in the absence of microtubules, but the sensing of chemical gradients of guiding cues is impaired and directional growth is lost (Williamson et al. 1996). The directionality of growth is determined by highly localised actin protrusion and

adhesiveness on one side of the growth cone against the other (Vitriol and Zheng 2012; Fig. 6.2c). This correlates with changes to the microtubule array: Microtubules are stabilised on the protruding and destabilised on the collapsing side, possibly through the action of APC (Buck and Zheng 2002; Zhou et al. 2004). One idea is therefore that microtubules direct the delivery of vesicles, mRNAs and GTPase activators to the growing side of the axon tip. The microtubule organisation in growth cones is dominated by front-directed microtubules that grow from the neurite into the growth cone (de Anda et al. 2005). Most of these microtubules do not extend all the way from the centrosome or Golgi network. Non-centrosomal microtubule nucleation occurs throughout the axon and dendrites (Stiess et al. 2010; Yau et al. 2014). In addition, severing enzymes such as Katanin or Spastin release microtubules from their anchoring at the centrosome, thereby enabling motor-driven transport of microtubules into neurites (Liu et al. 2010; Myers and Baas 2007; Yu et al. 2008). Advancing microtubules into the peripheral domain is then mediated by molecular motors of the kinesin-5 and kinesin-12 family (Nadar et al. 2008; Liu et al. 2010). In some migrating cells, the release of microtubules from the centrosome and cytoplasmic transport has also been observed, suggesting that similar mechanisms for microtubule reorganisation exist in migrating cells (Abal et al. 2002; Jolly et al. 2010).

In addition to the release of microtubules from their nucleation site, severing proteins also allow the destruction or amplification of microtubule subpopulations and can therefore modify the number of microtubules in a given orientation (Lacroix et al. 2010; Lindeboom et al. 2013; Sudo and Baas 2010). Cutting the microtubule lattice will produce two microtubules with the same orientation that either rapidly depolymerise or are stabilised and grow. Newly created minus ends are stabilised by CAMSAP family proteins. Depletion of CAMSAP2 results in a reduction in posttranslationally modified microtubules, cell polarity and directional cell migration (Jiang et al. 2014), suggesting that the stabilisation of non-centrosomal microtubules and the amplification of front-directed microtubules through collaboration of severing enzymes and minus end stabilisers are important for the asymmetric microtubule arrangement in motile cells.

Katanin localises to the leading edge of migrating human and Drosophila S2 cells and negatively regulates migration of these cells in vitro (Zhang et al. 2011). Katanin appears to be enriched at sites of filopodia formation (Liu et al. 2008), and increased amounts of Katanin subunits have been linked to more aggressive migratory behaviour in prostate cancer cells (Ye et al. 2012). Similarly, inhibition of Katanin subunits leads to migration impairment in mouse neurons and rat epithelial cells (Sudo and Maru 2008; Toyo-Oka et al. 2005). Uncontrolled function of Katanin and Spastin leads to aberrant numbers of microtubules in neurons, which has been linked to a number of diseases such as hereditary spastic paraplegia or Alzheimer's disease (Errico et al. 2002; Sudo and Baas 2011), causing general defects in microtubule-mediated transport.

In addition to increased nucleation of microtubules towards the front of the cell and potential amplification mechanisms by severing enzymes, differences in microtubule stability contribute to the asymmetry of the microtubule cytoskeleton.

Tubulin acquires posttranslational modifications in long-lived microtubules. In migrating cells, a front-directed accumulation of microtubules containing acetylated and detyrosinated tubulin is often observed (Gundersen and Bulinski 1988; Umeshima et al. 2007), suggesting that front-directed microtubules are stabilised, thus further exacerbating microtubule asymmetry.

It is thought that the asymmetry in the microtubule array allows preferential traffic of cargoes to the front of the cells (Fig. 6.3; Bachmann and Straube 2015). Important cargoes for cell migration are actin and Arp2/3 mRNA (Lawrence and Singer 1986; Mingle et al. 2005), post-Golgi carriers (Miller et al. 2009; Yadav et al. 2009) and recycling endosomes (Palamidessi et al. 2008). As posttranslational modifications of tubulin can serve as guidance cues for microtubule motor proteins, efficient front-directed transport can be achieved by a combination of increased number and selective stabilisation and modification of microtubules to the leading edge. Track selectivity has been demonstrated for kinesin-1s, kinesin-2s and dynein (Dixit et al. 2008; Sirajuddin et al. 2014) in vitro, and there is some evidence that this is also the case in cells (Cai et al. 2009; Ghosh-Roy et al. 2012; Huang and Banker 2012; Jacobson et al. 2006; Reed et al. 2006). In mature neurons, this property of kinesins to preferentially bind differentially modified tubulin is exploited to selectively target cargo specifically to axons or dendrites (Burack et al. 2000; Jenkins et al. 2012). Likewise, a preference for transport to and accumulation at the rear of migrating cells has been shown for the kinesin-3 Kif1C, which is negatively regulated by tubulin acetylation (Bhuwania et al. 2014; Theisen et al. 2012).

Posttranslational modifications of tubulin also regulate the activity of Katanin and Spastin and the binding affinities of microtubule-associated proteins (MAPs) such as Tau. While acetylation and polyglutamylation of tubulin increases severing activity, decoration of the microtubule lattice with Tau protects microtubules from severing (Lacroix et al. 2010; Sudo and Baas 2010). Abnormal regulation of Tau has been associated with disease progression, most notably with neurodegenerative diseases such as dementia (Lee and Leugers 2012). Thus complex feedback loops involving chemical modification and modification-sensitive MAPs modulate the asymmetric microtubule network in migrating cells.

6.3 Spatial Regulation of Microtubule Dynamics

As mentioned above, differences in microtubule dynamics at the front and rear of the cell contribute to the asymmetry in the microtubule organisation. Cells express an arsenal of microtubule regulators that tightly control the assembly and disassembly of microtubules (van der Vaart et al. 2009). In cells, microtubule catastrophe occurs almost exclusively at the cell cortex (Komarova et al. 2002), and microtubule stabilisation occurs through the close coupling of rescue and catastrophe events, holding microtubules in a dynamic captured state with short length fluctuations (Straube 2011; Straube and Merdes 2007). Microtubules are captured at the leading edge's cell cortex by a number of pathways, including EB1/APC/

mDia1, LL5beta/ELKS/CLASPs, IQGAP/CLIP-170 and Dlg (Akhmanova et al. 2001; Drabek et al. 2006; Kroboth et al. 2007; Kumar et al. 2009; Nakamura et al. 2001; Pfister et al. 2012; Schober et al. 2009; Watanabe et al. 2009a; Wittmann et al. 2004). Microtubule capture can be maintained for prolonged times resulting in stable microtubules leading to the front of the cell. These long-lived microtubules in turn acquire a number of posttranslational modifications. While detyrosination protects microtubules from depolymerases and severing enzymes (Peris et al. 2009; Roll-Mecak and Vale 2008), acetylation and poly-glutamylation recruit microtubule-severing enzymes (Lacroix et al. 2010; Sudo and Baas 2010). Microtubule severing close to the cell cortex can result in the release of a captured microtubule and is a mechanism that allows the spatial regulation of microtubule stability (Zhang et al. 2011).

The inactivation of the microtubule destabilisers stathmin and MCAK at the front of the cell by phosphorylation results in a gradient of increasing microtubule stability towards the front of the cell (Braun et al. 2014; Niethammer et al. 2004). Likewise, the interaction of microtubules with focal adhesion sites results in different outcomes at the front and rear of the cell: While microtubules are captured at adhesion sites in the front of the cell (Kaverina et al. 1998), catastrophe is induced when microtubules contact trailing adhesions (Efimov et al. 2007). While the mechanisms underlying these differences remain to be understood, it is clear that microtubule dynamicity is crucial for cell migration. Freezing dynamicity with low doses of Taxol and other microtubule-targeting agents so that the overall organisation is not perturbed impairs protrusion in fibroblasts, migrating neurons and growing axons (Dunn et al. 1997; Liao et al. 1995; Rochlin et al. 1996; Tanaka et al. 1995; Umeshima et al. 2007; Vasiliev et al. 1970). Furthermore, interference with the dynamicity of rear microtubules specifically leads to decreased rear retraction and changes to the time HeLa cells and CHO fibroblasts spent migrating (Ganguly et al. 2012). When the regional differences in microtubule dynamics regulation are removed by inhibition of MCAK or constitutive activity of Rac1, directional cell migration is severely reduced (Braun et al. 2014).

6.4 How Do Microtubules Influence Cell Migration?

6.4.1 Cell Shape, Polarity and Directionality

Directional cell migration requires the establishment of distinct regions in the cell as the front and the rear. This is often reflected in the morphology of the cell, where the leading edge is protruding either as a flat lamellipodium, using spiky filopodia, pseudopods or more complex structures such as the leading process of neurons. Retracting rears can be either (1) curved inwards pushing against the nucleus as in keratinocytes, (2) long, tail-like extensions as in some epithelial cells and fibroblasts or (3) uropods in leucocytes (Keren et al. 2008; Ratner et al. 1997; Theisen et al. 2012). In each configuration, the protruding edge, the nucleus and the retracting rear set up a single polarity axis. When branches or multiple protrusions

are formed, these are often used to make directional decisions in chemotaxis and neuronal pathfinding with the better-positioned protrusion persisting (Andrew and Insall 2007; Cooper 2013). Directional protrusions for cell migration are very similar to emerging axons. In some neurons such as cortical projection neurons, the axon is formed during cell migration by extending cell tails that continue to grow rather than retract (Cooper 2013).

Yet how is the polarity axis established? In cells that have been "starved" by serum withdrawal and then exposed to a chemical attractant gradient, a protruding extension is established towards the higher concentration of the chemical, and the cell begins to move up the gradient. A very similar mechanism guides axon growth cones along attractive or repulsive gradients (Vitriol and Zheng 2012). This mechanism has been conserved from amoeba to humans (Van Haastert and Devreotes 2004). Even in the absence of a guiding chemical gradient, cells from higher eukaryotes that are not surrounded by others spontaneously polarise and form a lamellipodium at one side of the cell. In keratinocytes, symmetry breaking occurs by contraction of actin filaments by non-muscle myosin II on one side of the cell, leaving the opposite side free to protrude (Yam et al. 2007). In epithelial cells, adhesion at the rear and formation of a tail precede protrusion in the opposite direction (Rid et al. 2005; Vicente-Manzanares et al. 2009). Pulling forces from other cells in a collective result in protrusion at the opposite cell edge, resulting in mechanical feedback and coupling of collective cell migration (Weber et al. 2012).

For a cell to change direction, either the polarity axis is gradually shifted, the cell depolarises and repolarises again in a new direction, or the front bifurcates or branches with one of the new protrusions taking over as front after a while (Petrie et al. 2009). The latter mechanism of branching and retraction of a branch is a pathfinding mechanism, for example, in migrating cortical interneurons and neocortical neurons (Cooper 2013; Sakakibara et al. 2014).

How do microtubules support the establishment, maintenance and changes of the polarity axis? As explained above, the asymmetry in the microtubule organisation and distribution of posttranslational modifications enables intracellular trafficking along microtubules to be asymmetric. Important cargo for cell polarity and migration is generated in and near the nucleus in the cell centre and requires transport along microtubules for delivery to the cell edges. An example is the mRNA for β-actin, which localises to the leading edge of migrating cells and is transported by kinesin-1 and dynein along microtubules (Kislauskis et al. 1997; Ma et al. 2011). The localised translation of actin mRNA is important for directional cell migration as it dictates the sites of actin filament nucleation (Katz et al. 2012). Equally importantly, proteins modified and packaged in the Golgi apparatus are transported efficiently to the leading edge via front-directed microtubules nucleated at the trans-Golgi by CLASPs (Miller et al. 2009). Further important cargoes to support front protrusion are vesicles that can be used as a source for additional membrane and supply receptors for adhesion helping protrusion at the leading edge (Etienne-Manneville 2013). It can be beneficial to distribute receptors for sensing chemical gradients and to adhere to the extracellular substrate and neighbouring cells

unequally at the cell surface to enhance or adapt to extracellular signals and regulate adhesion in different parts of the cell.

Given that the asymmetry in the microtubule cytoskeleton is key to directional intracellular transport, factors that regulate centrosome positioning such as Lis1 are implicated in developmental diseases due to impaired neuronal migration. Lis1 interacts with dynein to regulate the forces acting on cortical microtubule ends and thereby the centrosome and is crucial to moving the nucleus forward, an essential step in neuronal migration (Umeshima et al. 2007). The loss of Lis1 leads to a smooth brain surface, abnormal neuronal layering and large brain ventricles in humans (Ozmen et al. 2000; Pilz et al. 1998). Similar defects in brain morphology are caused by insufficient neuron migration upon loss of Dcx (doublecortin) (Gleeson et al. 1999a; Gleeson et al. 1999b; Liu 2011; Pilz et al. 1998). Dcx is a MAP that increases microtubule stability, but can also interact with Lis1 (Caspi et al. 2000). Centrosome position also determines the site of axon growth when hippocampal neurons differentiate (de Anda et al. 2005). It is thought that centrosome position again creates a bias of microtubules towards specific sites of the cells, with consequences for intracellular trafficking, protrusion, adhesion and signalling. In line with this idea, the amplification of centrosomes results in increased protrusion and invasion, probably by increasing front-directed microtubule activities (Godinho et al. 2014). For additional information on neurodevelopmental disorders caused by defective cell migration, please also consult the Chap. 5 by Gambarotto and Basto and the Chap. 4 by Sánchez-Huertas, Freixo and Lüders.

It is now firmly established that signalling by small GTPases of the Rho family is important in cell polarity (Nobes and Hall 1999). Small Rho GTPases are proteins that are active in the GTP-bound state, and their activity is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GAPs accelerate GTP hydrolysis to switch off the Rho GTPase, while GEFs accelerate the removal of the product and binding of GTP to activate Rho GTPase signalling. Once activated, Rho GTPases bind to a number of effectors such as protein kinases and actin-binding proteins (Sit and Manser 2011). In migrating cells, the most important Rho GTPases are Rac1, Cdc42 and RhoA. Their activity regulates cell polarity: Rac1 is most important in regulating the protrusion of cells through the WAVE and Arp2/3 complex (Eden et al. 2002). Cdc42 is most active at the cortical zone to promote protrusion via the WASP pathway and is important in orienting the centrosome towards the leading edge via the PAR complex, dynein and microtubules (Etienne-Manneville et al. 2005; Palazzo et al. 2001). RhoA is active further into the lamella and at the rear of the cell to regulate actin contractility (Amano et al. 2010; Machacek et al. 2009). Microtubules are known to influence the activity of Rho GTPases through the local distribution and function of GEFs, GAPs and effectors. Growing microtubules activate Rac1, while the release of microtubule-bound GEF-H1 upon microtubule depolymerisation activates RhoA (Nalbant et al. 2009; Ren et al. 1998); thereby, microtubule dynamics supports the localised activity of Rho GTPases. In turn, GTPases also influence microtubule stability in a positive feedback loop to improve cargo delivery to sites of active

protrusion, e.g. RhoA stabilises microtubules via IQGAP1 and mDia1 (Brandt et al. 2007; Kholmanskikh et al. 2006; Wen et al. 2004; Wittmann et al. 2004).

A second connection between microtubules and cell polarity is established through the interaction of microtubules with the Par complex (Suzuki and Ohno 2006). The Par complex, composed of Par6, atypical protein kinase C and Par3, regulates centrosomal polarity. The complex acts downstream of Cdc42 and regulates the activity of GSK3 kinases, which in turn control the activity of MAPs and thereby influence microtubule dynamics locally at the leading edge (Etienne-Manneville et al. 2005). A related protein, MARK/Par-1, can detach MAPs from microtubules to destabilise them (Ebneth et al. 1999; Tassan and Le Goff 2004). MARK activity is highest at the rear and lowest at the front of the cell, increasing the front-biased asymmetry in the microtubule array (Hayashi et al. 2012).

Recently, evidence is accumulating that maintaining an extended cell rear can influence persistent motility. The maintenance of such a tail requires adhesion at the rear despite high contractile forces. Reduction of contractile forces allows formation of extended tails in CHO cells and increases cell motility (Vicente-Manzanares et al. 2007). Likewise, microtubule transport of integrins into cell tails is required for the maturation of trailing focal adhesions and the stability of cell tails. Interfering with microtubule transport by depletion of the kinesin motor Kif1C results in shortened lifetime of cell tails and more frequent directional changes in migrating cells (Theisen et al. 2012). Similarly, drug treatments that suppress dynamic microtubules in the rear of the cell led to increased tail stability and affected directionality in HeLa and CHO cell (Ganguly et al. 2012). In these cells, the morphology of the front of the cells was not affected nor was the front-oriented position of the centrosome, arguing that the cells' ability to polarise was not globally perturbed. One hypothesis is that drag generated at the cell rear acts as a mechanical cue to support protrusion in the opposite direction (Theisen et al. 2012; Weber et al. 2012). Likewise, the extended cell polarity axis could facilitate biochemical gradients and cytoskeletal filament orientation (Rid et al. 2005; Theisen et al. 2012).

In sum, microtubules have important functions in supporting cell polarity by ensuring that signalling and actin-dependent processes are asymmetric. The interactions with the actin cytoskeleton are likely to function as a positive feedback loop, in which microtubules deliver actin-regulating proteins, while proteins localising to the actin cortex enhance microtubule stability (Siegrist and Doe 2007).

6.4.2 Force Generation

Forces generated by microtubules themselves are generally thought to be of minor importance for moving a cell forward. Microtubules can generate pushing and pulling forces through coupling polymer assembly and/or disassembly to subcellular structures. These forces are harnessed in the movement of chromosomes during mitosis and contribute to the distribution of the endoplasmic reticulum (Jordan and

Wilson 2004; Waterman-Storer and Salmon 1998). In cell types where only a small number of pioneer microtubules reach the plasma membrane, the direct contribution of microtubules to membrane protrusion is probably not significant. However, large numbers of microtubule ends reach the cell edge in axonal growth cones, and pushing forces generated by assembling microtubules are likely to be harnessed for cell protrusion (Liu et al. 2010). In this system, microtubule motors also generate forces either by sliding two microtubules relative to each other or by moving microtubules relative to the cell cortex, so that more microtubule ends reach the cortex. The main motors implicated in microtubule motility are kinesin-1 and dynein. Kinesin-mediated microtubule-microtubule sliding has been shown to generate forces for the protrusion of neurites (Lu et al. 2013; Myers and Baas 2007). To which extent forces generated by microtubule sliding and polymerisation directly contribute to cell migration remains to be established as microtubules also affect cell protrusion by a number of indirect pathways, most of which involve the actin cytoskeleton. It is well accepted that pushing forces generated by the assembly of actin at the cell front are the main driving force for cell protrusions. Likewise myosin-mediated contraction of actin bundles generates hydrostatic pressure and contractile forces involved in protrusion as well as contraction. Therefore, force generation during cell migration is primarily attributed to the action of the actin cytoskeleton.

Microtubules support actin-mediated cell protrusion indirectly through delivery of vesicles, i.e. lipids to the cell front, thereby allowing the expansion of the plasma membrane at the leading edge. The positioning of mRNA for actin and Arp2/3 at the leading edge is likely to involve microtubule-based transport and ensures a ready supply of actin monomers and the main actin nucleator for lamellipodial protrusion at the front of the cell (Jaulin and Kreitzer 2010; Mingle et al. 2005; Oleynikov and Singer 1998). Furthermore, the microtubule plus end complex contains a number of actin nucleators and regulators. Amongst them is adenomatous polyposis coli (APC), a protein that also promotes microtubule assembly (Kita et al. 2006; Mimori-Kiyosue et al. 2000) and acts as an actin nucleator in synergy with the formin mDia1 (Nathke et al. 1996; Okada et al. 2010). In addition to APC, a number of MAPs have been identified to bind and/or regulate both microtubules and actin. These include CLASPs, ACF7, MAP4 and dynein/dynactin (Matsushima et al. 2012; Rodriguez et al. 2003; Tsvetkov et al. 2007; Wu et al. 2008). For example, GSK3β acts downstream of the polarity-regulating GTPase Cdc42 and controls microtubule stability via ACF7 and other factors (Etienne-Manneville and Hall 2003; Kodama et al. 2003). ACF7 itself cross-links actin and microtubules, influences microtubule dynamics and has microtubule guidance functions (Applewhite et al. 2010; Wu et al. 2008). Also the non-receptor tyrosine kinase ABL2/Arg binds to microtubules and actin and promotes cell protrusion and spreading. This activity requires the physical coupling between F-actin and microtubules by ABL2 (Miller et al. 2004).

Other ways in which microtubules can influence actin polymerisation are by locally regulating small GTPase signalling, which in turn regulate force generation. It has been known for some time that microtubule polymerisation can activate Rac1 (Montenegro-Venegas et al. 2010; Waterman-Storer et al. 1999). Microtubules bind

the Rac1 activators Tiam1, Stef and Trio (Pegtel et al. 2007; Rooney et al. 2010; van Haren et al. 2014), thus allowing microtubule-dependent regulation of Rac1 through several pathways. RhoA can be activated by GEF-H1, which is sequestered on the microtubule lattice and activated upon release during microtubule catastrophe (Nalbant et al. 2009; Ren et al. 1998).

Thus a complex network of structural and signalling interactions between the microtubule and actin cytoskeleton at the cell front controls cell migration, and a fine balance between these activities is important for robust and directional cell migration (Kaverina and Straube 2011). So far, no diseases have been linked to an imbalance of forces in cell migration, but as many of the players involved serve multiple functions, and we do not yet fully understand how they interact with each other, it may be possible that we are underestimating the significance of a force imbalance for disease development. This intriguing area awaits further investigation, but individual players (e.g. APC, RASSF1A) have already been demonstrated to play important roles in cancer development (Humbert et al. 2008; Kassler et al. 2012; van Es et al. 2001).

While actin and non-muscle myosin II provide the forces necessary for protrusion at the leading edge, the microtubule cytoskeleton with its motor dynein can supplement these forces when necessary. In elongated cells that need to move in coherent clusters within surrounding tissue pressing in on them, such as migrating neurons, moving the nucleus presents a difficult challenge (Harada et al. 2014). The nucleus is the bulkiest organelle in the cell that cannot easily be compressed without causing DNA damage. Hence moving it against pressure from the environment requires forces that exceed those that actin rear contraction can provide (Tsai et al. 2007). The close spatial localisation of the centrosome to the nucleus in interphase cells has suggested early on that microtubules might be important in this task. Experiments on granule cells from mice explant cultures could demonstrate that stable microtubules and dynein are essential to move the nucleus and to position the centrosome in front of the nucleus (Tsai and Gleeson 2005; Umeshima et al. 2007). These results have led to two models on how microtubules and dynein can be used to move the nucleus (nucleokinesis): One model suggests that dynein is anchored at the leading edge to pull on plus ends of microtubules whose minus ends are embedded in the centrosome, which serves to translate the forces from dynein into net forward movement of the nucleus (Tsai and Gleeson 2005). Another model implicates a cage formed from a subpopulation of acetylated microtubules that encloses the nucleus and transmits the force generated by cortex-anchored dynein to move the nucleus forward (Umeshima et al. 2007). It should be noted though that not all neurons use dynein-mediated forces to move their nuclei. Differences exist between types of neurons and between the same neuron types in different organisms. For example, different force-generation models implicating actin-generated pushing forces exist for cerebellar Purkinje cells and cortical interneurons and also for cerebellar granule cells from mice and zebrafish (Cooper 2013). One possible explanation for these differences was proposed to lie in the different cell shapes, as the wider zebrafish cells might be able to move the nucleus

by actin-mediated contractility alone, while the very narrow and elongated mouse neurons require additional microtubule-mediated forces (Cooper 2013).

6.4.3 Adhesion

In order for the cell to move forward, the forces generated through actin polymerisation and contraction need to be transmitted to the extracellular matrix or neighbouring cells. To achieve this, cells form adhesive structures: focal adhesions and podosomes that attach to the extracellular matrix, and tight junctions, gap junctions and adherens junctions that link them to neighbouring cells. The size and composition of these structures depend on the type of cell and the cellular environment. Typically, adhesive structures are formed by a transmembrane receptor, which contacts the substrate on the outside of the cell or forms homophilic interactions with the neighbouring cells. The receptor is then stabilised on the inside of the cell by association with other proteins. The adhesion complexes are connected to the cytoskeleton, which will also contribute to clustering of such complexes into larger structures.

The dependence of cells on adhesion for migration can be very different. In confined environments, protrusions such as blebs can generate enough traction themselves to allow the cell to move forward efficiently. Pressurised blebs can be used to find the weakest linkage between cells and can create a foothold for moving cells trying to cross tissues (Lammermann and Sixt 2009; Mandeville et al. 1997; Sanz-Moreno and Marshall 2010; Wolf et al. 2003b). Such modes of migration are employed by cells of the immune system, such as neutrophils and leucocytes, and some tumour cells (Friedl et al. 1998a; Friedl et al. 1998b; Werr et al. 1998). Mesenchymal migration of fibroblasts and epithelial cells relies strongly on cell adhesion for migration in 2D as well as in 3D (Sanz-Moreno and Marshall 2010). Adherent cells can use different classes of receptors to attach to their surroundings; the classic receptors for a variety of extracellular matrix molecules are integrins. Integrins are obligatory heterodimers of an α- and a β-chain, and different combinations of the 18 α- and 8 β-chains in mammalian cells result in 24 different receptors with distinct substrate specificity (Hynes 2002). Integrins are embedded in the plasma membrane with the greater part of the protein extending into the extracellular space where it directly binds to matrix proteins. Exocytosis of integrin-containing vesicles delivered by microtubule-dependent trafficking occurs at the leading edge (Bretscher and Aguado-Velasco 1998; Spiczka and Yeaman 2008) allowing the formation of small focal complexes. At least in part, this process is controlled by Rac1 which becomes activated by Tiam2, which in turn is regulated by microtubules (Rooney et al. 2010). Focal complexes turn over rapidly with only a few of them maturing into focal adhesions. Focal adhesions consist of >150 proteins on the cytoplasmic side, which mediate links to actin fibres and/or function in signalling (Zaidel-Bar et al. 2007). Focal adhesion maturation is force dependent: Actin contractility increases the size of adhesions as well as the density of adhesion

molecules in the adhesion (Parsons et al. 2010). This response allows adhesion strength to scale to the forces applied to them.

Microtubules are important regulators of focal adhesions. The disassembly of microtubules by small-molecule inhibitors results in the formation of large focal adhesions, while their turnover is induced as soon as microtubule regrowth is permitted by washing out of the drug (Ezratty et al. 2005; Waterman-Storer et al. 1999). Furthermore, microtubules have been observed to target focal adhesions repeatedly with their dynamic plus ends, and this targeting results in the dissolution of focal adhesions (Kaverina et al. 1999; Kaverina et al. 1998; Krylyshkina et al. 2003; Rid et al. 2005). Microtubules are thought to reach focal adhesions by guidance along actin filaments. In migrating fibroblasts, microtubules are crossbridged to actin filaments by a number of factors including ACF7, IQGAP1/CLIP-170 or CLASPs, which then guide the growing microtubule ends to focal adhesions (Drabek et al. 2006; Small and Kaverina 2003; Stehbens and Wittmann 2012). Microtubule ends reduce their growth speed and undergo catastrophe upon contact with focal adhesions. This process is regulated by paxillin, a structural component of focal adhesions (Efimov et al. 2008). Often, the microtubule undergoes a rescue and targets the same or another focal adhesion, thereby resulting in the repeated targeting of adhesions and their turnover.

One possible way how microtubules could disassemble focal adhesions is by interacting with signalling molecules that control the composition of focal adhesions (Etienne-Manneville 2013; Wickstrom et al. 2010), and another is that microtubules deliver components of the endocytic machinery, as could be shown for dynamin and Clathrin, to help internalise integrins for recycling (Chao and Kunz 2009; Ezratty et al. 2009; Nishimura and Kaibuchi 2007). Also, microtubule-dependent control of the local release of proteases into the extracellular space may promote the detachment of the cell from the substrate by cleaving substrate-bound receptors (Takino et al. 2006). It was demonstrated that exocytosis of such proteases occurs in the vicinity of focal adhesions (Steffen et al. 2008; Wiesner et al. 2010), but if this mechanism plays a role in cell migration remains to be established (Margadant et al. 2011). It is, however, well known that localised secretion of metalloproteases is important for the migration of cancer cells through existing tissue (Hegerfeldt et al. 2002; Takino et al. 2006; Wang and McNiven 2012; Yilmaz and Christofori 2009). Blocking these proteases stops the migration of fibrosarcoma and mammary carcinoma cells (Coopman et al. 1998; Wolf et al. 2003a). Likewise, microtubule-dependent regulation of actin dynamics (see section above) could affect the force coupling into focal adhesions with loss of the pulling force resulting in the dissolution of the focal adhesion.

The microtubule-dependent control of focal adhesions requires motor-dependent transport as kinesin-1 has been demonstrated to be required for the process (Krylyshkina et al. 2002). Podosomes, invasive adhesion structures prevalent in immune cells such as macrophages and dendritic cells, require the kinesin-3 Kif1C for their formation and dynamic turnover and Kif9 for their function in matrix degradation via localised exocytosis (Bachmann and Straube 2015; Cornfine et al. 2011; Efimova et al. 2014; Kopp et al. 2006). However, it is currently not

clear which cargoes are delivered by these kinesins that contribute to the observed processes.

Controlled turnover of focal adhesions is likely to play a role in the metastatic behaviour of cancers, regulating the aggressiveness of disease progression by the cells' motility and invasiveness (McLean et al. 2005; Recher et al. 2004). The formation of adhesions is in the range of several minutes, which can be the rate-limiting step in migration as shown by the increase in cell migration speed in vinculin-depleted cells (Friedl et al. 2004; Mierke et al. 2010). In accordance with this, a reduction in cell adhesiveness has been implicated in the progression of cancer (Sanz-Moreno and Marshall 2010). Cells migrating as collective, either as clusters of cancer cells or during developmental processes, need to maintain close connections to the other cells at all times in order to improve their migration efficiency, as surrounding tissues pose significant obstacles. Cadherins play an important role in this.

Cadherins are a large family of membrane-bound receptors that form homophilic interactions with molecules on the surface of neighbouring cells. This establishes a tight link between cells. Examples of cells that depend on N-cadherin for motility are a number of different types of migrating neurons (Jossin and Cooper 2011; Lele et al. 2002; Monier-Gavelle and Duband 1995; Nakagawa and Takeichi 1998; Rappl et al. 2008; Rieger et al. 2009) but also cells forming the lateral line organ in zebrafish (Revenu et al. 2014) and cancer cells (Qi et al. 2006; Shih and Yamada 2012). Other cells rely on E-cadherin, such as fibroblasts and keratinocytes (Maretzky et al. 2005). The increased cohesion mediated by cadherin within the cell cluster could facilitate pulling of follower cells along the path that the leader cells have created by breaking down the extracellular matrix (Friedl and Gilmour 2009), or it could provide a point of strong attachment for cytoskeletal elements to help move cell organelles like the nucleus forward, especially in neurons (Rieger et al. 2009; Tsai and Gleeson 2005). Like most other plasma membrane-bound proteins, cadherins require kinesin-based transport to reach their destination (Chen et al. 2003; Kawauchi et al. 2010; Mary et al. 2002; Yanagisawa et al. 2004). In addition, the plus ends of non-acetylated microtubules have been shown to cluster cadherins in the plasma membrane, a prerequisite to forming stable cell-cell connections (Stehbens et al. 2006; Waterman-Storer et al. 2000). Similar to cadherins, CAMs are a large group of proteins that can form homophilic interactions to connect two cells. They are often upregulated when cells obtain increased motile characteristics such as during metastasis (Lehembre et al. 2008; Schreiber et al. 2008). They possess functions in addition to adhesion, such as sensing chemical gradients during migration, making their regulation even more complex (Cavallaro et al. 2001; Francavilla et al. 2007; Paratcha et al. 2003; Yilmaz and Christofori 2009).

All these different types of adhesions have their own signalling pathways, which link adhesions and their various states of engagement to polarity signalling and microtubule stability, and they all depend on microtubule-based transport from the cell centre to the surface. This places microtubule-mediated transport at the centre of the regulation of local adhesiveness by site-directed delivery of substrate

receptors or regulatory elements (Miller et al. 2009; Yadav et al. 2009). Many cell surface proteins have residency times at the surface in the range of seconds to minutes (Bretscher 2008), before they need to be internalised and either transported back into the cells for processing or returned to specific sites to counteract diffusion in the plasma membrane. For N-cadherin and α5β1 integrin, for example, recycling pathways have been described which can be rather elaborated, involving internalisation, retrograde transport to recycling compartments that can be as far away as next to the centrosome and return to the surface (Bretscher 1989; Caswell and Norman 2008; Gu et al. 2011; Shieh et al. 2011). Through their transport capacity and motor protein preference for specific microtubule tracks, cargo can be directed to different parts of the cell (Cai et al. 2009; Reed et al. 2006), giving microtubules control over the amount and position of adhesive complexes on the cell surface. For example, Kif1C transports integrin-containing vesicles in migrating cells. This transport is required for the maturation of focal adhesions in the rear of the cell as it provides the ready supply of integrins for additional incorporation and exchange. A reduced supply of surface integrin results in a misbalance of contractile forces and adhesion strength causing the frequent contraction of cell tails and loss of polarity (Theisen et al. 2012). Recently, kinesins Kif15 and Kif4A have also been implicated in integrin transport (Eskova et al. 2014; Heintz et al. 2014). How the different transport pathways contribute to the microtubule-dependent regulation of cell adhesion remains to be elucidated.

6.4.4 Signalling

The coordination of the cell migration machinery at the front and rear of the cell and the response to environmental signals and guidance cues involve complex signalling networks. Amongst the well-characterised pathways organising migration are polarity signalling (small GTPases), adhesion signalling (integrins and cadherin) and guidance signalling (with the use of second messengers, intracellular calcium and phosphoinositol species).

Rho GTPases regulate actin dynamics, contractility and cell adhesion (Sit and Manser 2011). Rho GTPase signalling pathways are spatially restricted allowing the local regulation of protrusion and retraction enabling cell migration and other processes such as cytokinesis, phagocytosis and morphogenesis (Hall 2012). Microtubules control Rho GTPases signalling (1) by delivery of GTPases Rac1 and Cdc42 to the membrane (Osmani et al. 2010; Palamidessi et al. 2008); (2) by positioning GEFs such as Tiam1, Stef/Tiam2, Trio and effectors such as IQGAP1 (Briggs et al. 2002; Briggs and Sacks 2003b; Rooney et al. 2010; van Haren et al. 2014); and (3) by sequestering GEFs and coupling their release and activation to microtubule dynamics such as GEF-H1/RhoGEF2 (Chang et al. 2008; Glaven et al. 1999; Krendel et al. 2002; Rogers et al. 2004). In turn, Rho GTPases regulate microtubule dynamics. In cells without the Rac1 GEF Tiam1, microtubules are unstable (Pegtel et al. 2007), and Cdc42 influences the polarity of the microtubule array via the Par complex and GSK3β (Etienne-Manneville et al. 2005; Watanabe

et al. 2009a). Therefore, the relationship between microtubules and GTPases is balanced by feedback loops (for further examples, see review by Etienne-Manneville 2013).

Rho GTPase signalling is connected to adhesion signalling. Cadherins at the plasma membrane are signalling hubs via their binding of β-catenin and p120. β-catenin can be released from cadherin to move into the nucleus and, as co-factor, triggers the transcription of several genes, including those of adhesion molecules (McCrea et al. 2009). This is a crucial event in Wnt signalling, a pathway that is often enhanced in cancer cells and metastasis and which is controlled by Cdc42 (Aman and Piotrowski 2008; Clevers 2006; Fukata et al. 1999; Heuberger and Birchmeier 2010). Another component of the Wnt signalling pathway is APC, which is localised at the leading edge at microtubule plus ends (Matsumoto et al. 2010; Okada et al. 2010) and which regulates β-catenin levels (Munemitsu et al. 1995). The release of β-catenin from cadherin is also partly depending on IQGAP1, which is an effector of Rho GTPases and can bind microtubules directly to stabilise them (Fukata et al. 1999; Fukata et al. 2002). p120, another catenin family protein normally found associated with cadherin, has been reported to suppress RhoA and increase the activity of Rac1 and Cdc42 to regulate cell-cell contacts and may be able to influence microtubule dynamics (Ichii and Takeichi 2007; Watanabe et al. 2009b).

Another example for crosstalk between polarity signalling and adhesion signalling is the relationship between small GTPases and integrin. Integrin signalling is activated by binding of integrins to the extracellular substrate and is mostly mediated through focal adhesion kinase (FAK) and integrin-linked kinase (ILK) (Schwartz 2001). FAK regulates the turnover of focal adhesions but also activates RhoA and mDia (Palazzo et al. 2004; Webb et al. 2004). As mDia can bind to microtubule plus ends at the leading edge, this could explain the observed link between FAK activity and microtubule stabilisation (Palazzo et al. 2004). Focal adhesions can also influence the activity of Cdc42, which can act back on microtubule stability (Etienne-Manneville and Hall 2001). In migrating neurons, interfering with the function of FAK leads to a disorganised microtubule array and defective nuclear movement, a prerequisite for neuronal migration (Xie et al. 2003). Similarly, ILK regulates Rac1 and therefore lamellipodium formation via its interaction partners α- and β-parvin (Legate et al. 2006; Zhang et al. 2004). ILK and microtubules together function to impart polarity on epithelial cells, and ILK is needed to organise microtubules in this system (Akhtar and Streuli 2013). Other effects of ILK include the regulation of microtubule dynamics through the interaction with IQGAP1 and mDia1 (Wickstrom et al. 2010).

Recently, it was proposed that local intracellular calcium levels, a second messenger common to many signalling pathways, could be another mechanism to coordinate the different signalling pathways and biological processes (Tsai et al. 2014). Calcium waves at the front of migrating fibroblasts dictate cell speed. As some of the microtubule-regulating proteins such as IQGAP1 require calmodulin and/or calcium for their function (Briggs and Sacks 2003a), it is possible that other signalling pathways which we currently do not know can

influence microtubules by these means indirectly and thereby increase the microtubule-centred regulatory network during migration.

6.5 Conclusion

While many cell types are able to generate movement in the absence of microtubules by employing their actin cytoskeleton, microtubules are consistently important in fine tuning several aspects of migration, such as establishing polarity, exercising spatial control over force generation and adhesion, as well as signalling. Microtubules span the entire cell, making it possible to coordinate these tasks across spatially distant cellular regions. Due to their intrinsic dynamic instability, microtubules can adapt quickly in response to external and internal cues.

Over recent years, it has become clear that imbalance or mis-regulation of microtubule dynamics and/or motor function can lead to disease or promote disease progression when cells that should move cannot (e.g. immune cells or cells in embryonic development) or cells that should not move gain the ability to break down tissue barriers and colonise other tissues (e.g. cancer metastasis). Further research will continue to elucidate the details of the molecular interactions and will help us to understand the development of diseases affecting many patients.

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