Structural investigations into functional aspects of the centriolar key proteins CEP135/Bld10p and SAS-6/Bld12p

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presented by

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I dedicate this thesis to my wife Patricia A. I. M. Jaaks
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<th>full name</th>
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<tbody>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>Alp14</td>
<td>altered polarity 14</td>
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<tr>
<td>Ana-2</td>
<td>anastral spindle 2</td>
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<tr>
<td>ANM</td>
<td>Anisotropic network model</td>
</tr>
<tr>
<td>APC/C</td>
<td>anaphase-promoting complex/cyclosome E3 ubiquitin ligase</td>
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<td>ASPM</td>
<td>abnormal spindle microtubule assembly</td>
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<td>AUC</td>
<td>analytical ultracentrifugation</td>
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<td>Aurora-A</td>
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<td>Bld12p</td>
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<td>calmodulin-regulated spectrin-associated protein</td>
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<tr>
<td>CASC5</td>
<td>cancer susceptibility candidate 5</td>
</tr>
<tr>
<td>CCP</td>
<td>cytosolic carboxy peptidases</td>
</tr>
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<td>CD</td>
<td>circular dichroism (CD) spectroscopy</td>
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<td>cdh1</td>
<td>APC/C activator protein CDH1</td>
</tr>
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<td>CDK</td>
<td>(cyclin dependent kinase)</td>
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<td>CNN</td>
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<td>CPAP (CENPJ)</td>
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<td>pericentrin-like protein <em>(Drosophila)</em></td>
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<td>chicken bursal lymphoma cell line</td>
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<td>electron microscopy</td>
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<td>FBF1</td>
<td>Fas (TNFRSF6) binding factor 1</td>
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<td>FP</td>
<td>fluorescence polarization</td>
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<td>GCN4</td>
<td>amino acid starvation-responsive transcription factor GCN4</td>
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<tr>
<td>GDP</td>
<td>guanosine-5'-diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine-5'-triphosphate</td>
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<td>histone deacetylase 6</td>
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<tr>
<td>HeLa</td>
<td>Henrietta Lacks</td>
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<tr>
<td>IMPLICO</td>
<td><em>Latin:</em> to entangle</td>
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<tr>
<td>IPTG</td>
<td>isopropyl 1-thio-b-galactopyranoside</td>
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<td>ITC</td>
<td>isothermal titration calorimetry</td>
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<td>Kif3a</td>
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<td>microtubule associated proteins</td>
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<td>MCM5</td>
<td>minichromosome maintenance complex component 5</td>
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<td>MCPH</td>
<td>autosomal recessive primary microcephaly</td>
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<td>NFAp</td>
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<td>Term</td>
<td>Definition</td>
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<td>------</td>
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<td>NPM/B23</td>
<td>nucleophosmin (nucleolar phosphoprotein B23, numatrin)</td>
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<td>P53</td>
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<td>PCH1 (VRK1)</td>
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<td>PCM</td>
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<td>Rac1</td>
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<td>Sak kinase</td>
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<td>SAPK</td>
<td>stress-activated protein kinase kinase kinases</td>
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<td>SEL-10</td>
<td>suppressor/enhancer of lin-12 10</td>
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<td>SFC</td>
<td>SKP1-cullin-F-box</td>
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<td>Sirt2</td>
<td>NAD-dependent deacetylase sirtuin-2</td>
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<td>Slimb</td>
<td>supernumerary limbs</td>
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<tr>
<td>Spd-2</td>
<td>spindle defective 2</td>
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<tr>
<td>SPICE1</td>
<td>spindle and centriole associated protein 1</td>
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<tr>
<td>STAN</td>
<td>Stil/A2a motif</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>----------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>STED</td>
<td>stimulated emission depletion</td>
</tr>
<tr>
<td>STIL</td>
<td>SCL/TAL1 interrupting locus</td>
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<tr>
<td>TIM</td>
<td>truncated in microcephaly</td>
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<tr>
<td>TOG</td>
<td>tumor overexpressed gene</td>
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<tr>
<td>TTL</td>
<td>tubulin tyrosine ligase</td>
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<td>tubulin tyrosine ligase-like proteins</td>
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<td>U2OS</td>
<td>human osteosarcoma</td>
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<td>WDR62</td>
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<td>ZNF335</td>
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<td>α-Tat1</td>
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<tr>
<td>βTrCP</td>
<td>beta-transducin repeat containing E3 ubiquitin protein ligase</td>
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<tr>
<td>γ-TuRC</td>
<td>γ-tubulin ring complex</td>
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Summary
The centrioles are major constituents of the centrosome. They act as basal bodies for cilia and flagella and their disruption is thus implicated in many ciliopathic diseases. In general, two centrioles per cell are arranged perpendicular to each other in the centrosome. The canonical centriolar architecture consists of a 9-fold symmetric centriole wall generated by nine microtubule multiplets forming a hollow cylinder. In the proximal lumen resides a cartwheel-like structure, which contains a central ring-like hub from which nine spokes radiate outwards and connect to the microtubule multiplets.

Together with further centrosomal components, the centrioles follow their own duplication cycle, ensuring that both daughter cells inherit a set of mother and daughter centrioles. During the S phase, daughter centrioles start to form perpendicular to the existing centrioles. Several key proteins central to this duplication process and important for centriole architecture have been identified. However, only in recent years structural investigations started to reveal the underlying structure-function relationships. The centriolar protein SAS-6/Bld12p was found to constitute major parts of the cartwheel, including the central hub and main parts of the spokes. It was shown that SAS-6/Bld12p can assemble into 9-fold symmetric cartwheel-like oligomers in vitro. Therefore, it was hypothesized that the self-assembly property of SAS-6/Bld12p underlies the centriolar 9-fold symmetry. Nevertheless, whether the 9-fold symmetry is exclusively rooted in the SAS-6 self-assembly property and which key protein links the cartwheel spokes to the centriolar microtubule wall to enforce the symmetry on the centriole wall remained to be investigated. The centriolar protein CEP135/Bld10p was hypothesized to be the connecting link between SAS-6/Bld12p and the centriole wall. In addition, it was further shown that CEP135/Bld10p is central to the maintenance of the centriole integrity. A microtubule binding domain is essential to fulfill both functions directly. Indeed, microtubule binding was attributed to the N-terminal domains of human and Drosophila CEP135.

In this study we were able to localize the microtubule binding site of human CEP135 in the residue range 96-108 and identified three lysines important for microtubule binding. We further obtained a full model of the N-terminal domain of human CEP135, by combining X-ray crystallography, small angle X-ray scattering and molecular dynamics simulation. To the best of our knowledge, our crystal structure of the microtubule binding coiled-coil domain of CEP135 represents the first structure of CEP135/Bld10p and of microtubule binding coiled-coils in general. In addition, by introducing point mutations in
the N-terminal dimerization interface of SAS-6/Bld12p we achieved an alteration of the SAS-6/Bd12p assembly symmetry. Introducing selected mutations into human and *C. reinhardtii* cells resulted only in minor changes of the centriole symmetry. Only upon truncation of Bld10p in *C. reinhardtii*, which supposedly severed the physical connection between cartwheel and centriole wall, an alteration of cartwheel symmetry was observed. Therefore, we could demonstrate that an additional mechanism must contribute to the control of the centriolar 9-fold symmetry.
Zusammenfassung


In der vorliegenden Studie konnten wird die genaue Lage des mikrotubuli-bindenden Motivs auf dem humanen CEP135 bestimmen (Aminosäuren 96-108), sowie drei Lysine
Chapter 1
Introduction
1.1. The centrosome

1.1.1. Centrosome function and build up

The centrosome was first described by Van Beneden and Boveri during the 1880's (reviewed in Debec, Sullivan, & Bettencourt-Dias, 2010). It is a cell organelle implicated in cell division, cilia formation, cytoskeleton organization and signaling and follows its own tightly controlled duplication cycle. During the G1 phase of the cell cycle cytosolic microtubules are nucleated and anchored at the centrosome. After duplication during the S phase the two centrosomes become vital parts of the two spindle poles ensuring the organization of the bipolar spindle apparatus during mitosis. Although it is still controversial to which extent bipolar cell division can be achieved without centrosomes (reviewed in Hinchcliffe, 2014), dysregulation of the centrosome clearly affects the cell division (see Chapter 1.1.2. Centrosome disease implications).

The second major function of the centrosome is fulfilled by its sub-component the centrioles. During the G1 phase they can migrate to the cell cortex, where the older nascent centriole docks to the membrane and serves as a basal body for the formation of cilia and flagella (reviewed in Avasthi & Marshall, 2012; Dawe, Farr, & Gull, 2007). During migration the nascent centriole interacts with membrane vesicles from the Golgi. These vesicles help to establish the distinct flagella membrane. Although continues with the cell membrane, the flagella membrane is of distinct lipid composition (reviewed in Avasthi & Marshall, 2012). The so called ciliary vesicle will grow by addition of further vesicles until it fuses with the plasma membrane (reviewed in Yee & Reiter, 2015). After docking to the membrane the outgrowth of the outer microtubule doublets starts from the transition zone at the distal end of the microtubule triplets of the basal body (Ishikawa and Marshall, 2011). This is achieved by a complex system of intra-flagella transport, which delivers the required components to the growing distal cilia tip. Before being reused as a centriole in a further cycle of cell division the basal body cilia needs to be separated from the cilia either by resorption of the cilia (Marshall & Rosenbaum, 2001) or by deflagellation e.g. by katanin severing of the axoneme microtubule doublet in the transition zone (Parker et al., 2010).

The centrosome can serve those important cellular functions due to its complex modular architecture. It is canonically described as two cylindrical structures in perpendicular orientation, the centrioles, surrounded by a diverse matrix of proteins termed the pericentriolar material (PCM) (Figure 1.1.). The centrioles are roughly 200 nm in diameter and 400 nm in length and display a conserved 9-fold symmetry. They are
assembled around a proximal cartwheel. The nine spokes of this cartwheel radiate outwards from a central hub and are attached to the centriole wall consisting of microtubule multiplets (reviewed in Azimzadeh & Marshall, 2010; Gönczy, 2012; Hirono, 2014). The centriolar microtubule wall originates from a γ-tubulin ring complex (γ-TuRC) (Dammermann et al., 2004; Guichard et al., 2010). The architecture of the cartwheel and the centriole wall will be discussed in more detail in Chapter 1.2.

In the G1 phase the two centrioles lie perpendicular to each other and are loosely connect by a fibrous linker (Bornens et al., 1987) consisting of C-Nap1 (sodium channel and clathrin linker 1) and rootletin, whose depletions led to premature centrosome separation (Fry et al., 1998). While rootletin is suggested to be the physical linker and is spanning the gap between the centrioles (Yang et al., 2006, 2002), C-Nap1 is located at the proximal end of the centrioles and binds rootletin (Yang et al., 2006). C-Nap1 further forms a complex with CEP68 (centrosomal protein 68 kDa) and centlein which was shown to be essential for maintenance of cohesion (Fang et al., 2014).

The nascent centriole bears appendages at distal and subdistal positions (Anderson, 1972). The distal appendages are responsible for anchoring the centriole to the plasma membrane during its transformation into a basal body and are formed by sequential recruitment of several key proteins such as CEP83 (centrosomal protein 83 kDa), CEP89 (centrosomal protein 89 kDa), CEP164 (centrosomal protein 164 kDa), SCLT1 (sodium channel and clathrin linker 1), and FBF1 (Fas (TNFRSF6) binding factor 1) (Ishikawa et al., 2005; Tanos et al., 2013). Another important protein is CEP123 (centrosomal protein 123
kDa), whose depletion prevents the formation of the ciliary vesicles. CEP123 anchors via its C-terminus to the distal appendages and was found to interact with further distal/subdistal appendage proteins (Sillibourne et al., 2013).

The subdistal appendages are stabilizing anchor points for cytosolic microtubules (reviewed in M. Bornens, 2012; Tateishi et al., 2013). Several key components of the subdistal appendages have been identified such as ninein, centrolin, cenexin/ODF2 (outer dense fiber of sperm tails 2), ε-tubulin, Kif3a (Kinesin family member 3a), CEP170 (centrosomal protein 170 kDa) and p150 (Dynactin subunit p150) (Chang et al., 2003; Gromley et al., 2005; Ishikawa et al., 2005; Kodani et al., 2013; Lange, 1995; Mogensen et al., 2000; Nakagawa et al., 2001). For many of those proteins it was demonstrated that they are vital for the anchoring of microtubules at the centrosome and therefore are indispensable for centrosomal function. For instance, mouse ninein was shown to localize to the centriole via its C-terminus and to γ-tubulin via its N-terminus. Thus ninein may be the link between microtubule nucleation and anchoring (Abal et al., 2002; Delgehyr et al., 2005). Human ninein isoforms were shown to comprise a γ-tubulin binding site towards the C-terminus in their coiled-coil region, which also harbors two of the four centrosome targeting sites in ninein (Lin et al., 2006).

Kif3a was suggested to recruit p150glue to the subdistal appendages, which in turn would recruit further components such as ninein and CEP170 (Kodani et al., 2013). ODF2 on the other hand was shown to be an important component for appendage formation in general. While distal appendage formation only requires the C-terminal domain, subdistal appendage formation requires the N- and C-terminal domain of ODF2. Furthermore, ODF2 was hypothesized to be an organizing factor for both types of appendages. Interestingly, basal feet and transition fibers in basal bodies are likely homolog to subdistal and distal appendages (Tateishi et al., 2013).

The surrounding PCM was formerly described as amorphous material (reviewed in Lüders, 2012). However, recent studies in human cell lines (Lawo et al., 2012; Sonnen et al., 2012) and in Drosophila (Fu and Glover, 2012; Mennella et al., 2014, 2012) revealed an internal structure for the PCM. Its components form toroid layers around the proximal end of the mother centriole (Figure 1.2.).
Long elongated proteins such as pericentrin (Lawo et al., 2012) and CEP152/Asl (centrosomal protein 152 kDa/asterless) (Mennella et al., 2012) span the toroid ring radially like spokes with their C-terminus oriented towards the microtubule wall. The other major components are arranged as layers in the toroid ring. CEP120 (centrosomal protein 120 kDa) is located closest to the centriole wall. Furthermore, the orthologs CEP192 (centrosomal protein 192 kDa, human) and Spd-2 (spindle defective 2, Drosophila) are distributed homogeneously in the inner toroid layer around the centriole wall. The outer toroid layers are formed by NEDD1 (neural precursor cell expressed, developmentally down-regulated 1), γ-TuRC/γ-tubulin and CDK5RAP2 (CDK5 regulatory subunit associated protein 2) complexes (Lawo et al., 2012), which are responsible for the nucleation of cytosolic microtubules. During the onset of mitosis at the G2 to M transition, the kinase Plk1 recruits further PCM material, the centrosome size increases and an additional PCM matrix is formed around the PCM toroid layers (reviewed in Lüders, 2012; Mennella et al., 2014). These additional matrix elements consist of complexes of NEDD1, γ-TuRC/γ-tubulin and CDK5RAP2 with pericentrin and show the same internal spatial arrangement (Lawo et al., 2012). Albeit this additional material is not radially orientated but forms a matrix around the toroid ring. The recruitment of the additional matrix is Plk1 (Polo-like kinase1) dependent. Plk1 phosphorylates pericentrin which in turn leads to the recruitment of NEDD1, CEP192, Aurora-A (aurora kinase A) and γ-TuRC (Conduit et al., 2014, 2010). It was reported by Fu and Glover that the CDK5RAP2 ortholog CNN (centrosomin, Drosophila) is responsible for the recruitment of γ-TuRC and depends on Plk1 for its recruitment.
localization to the centrosome (Fu and Glover, 2012). However, the accumulation of CDK5RAP2 (human), which is the first component to accumulate in the growing matrix, seems to be caused by pericentrin (PCNT) independent of pericentrin phosphorylation by Plk1 (Lee and Rhee 2011). For the centrosomal targeting of the γ-TuRC complexes NEDD1 was found to be required (Haren et al., 2009). Furthermore, pericentrin may be sufficient for recruitment of γ-TuRC in the absence of CDK5RAP2 (Lawo et al., 2012). Interestingly, during the centriole duplication in the G2 phase the toroid layers and the matrix around the mother centriole display a gap were the daughter centriole emerges (Lawo et al., 2012; Mennella et al., 2012; Sonnen et al., 2013).

Distributed in the PCM are distinct granular bodies termed centriole satellites. Their major role is thought to be the proteostasis of the centrosome by guiding the transport of required components and thereby regulating the supply and localization of proteins involved in ciliogenesis and centrosome maturation (reviewed in Tollenaere, Mailand, & Bekker-Jensen, 2015).

Although the centrosome is an organelle exclusive to eukaryotes (reviewed in Ross & Normark, 2015) it is found only in animals and some fungi. Centrioles however, are not only a substructure of the centrosome but can exist independent of the centrosome. They are conserved throughout all eukaryotic clades, apart from a few exceptions (Carvalho-Santos et al., 2011). Since centrioles are not only the central component of the centrosome but also serve as scaffold in basal body formation, it is assumed that the formation of basal bodies is the more ancestral function of centrioles (Bornens and Azimzadeh, 2007). In a study by Hodges et al. only 2 out of 13 centrosome function specific proteins were found to exist outside of the holozoa (Hodges et al., 2010). The authors conclude that the centrioles of the eukaryotic cenancestor fulfilled basal body function but were not associated with the centrosome.

1.1.2. Centrosome related diseases
As discussed previously, the centrosome mainly serves as the microtubule organizing center and its centrioles provide the essential basal bodies for ciliogenesis. Not surprisingly dysfunction of the centrosome and its components are linked to a vast range of diseases. Foremost ciliopathic diseases, cancer and diseases related to abnormal cell division are associated with centrosome and basal body defects.

Aneuploidy has been described as a central element of cancer (reviewed in Weaver...
& Cleveland, 2006). In general, chromosome instability gives rise to aneuploidy and is in turn influenced by centrosome dysfunction (reviewed in X. Fang & Zhang, 2011). Chromosome aneuploidy can be rooted in chromosome missegregation. Causes for such missegregation are either a failure in the spindle assembly checkpoint signaling, the microtubule-kinetochore attachments or abnormal mitotic spindles (reviewed in Zyss & Gergely, 2009). Due to its prominent position in cytokinesis it was hypothesized by Boveri that centrosome aberrations favor tumor progression (reviewed in Hansford & Huntsman, 2014).

Nevertheless, it is an ongoing debate whether centrosome dysfunction is a cause of cancer progression or just the result of mitotic spindle abnormalities in cancer cells (reviewed in Bettencourt-Dias & Hildebrandt, 2011; Nigg & Raff, 2009; Zyss & Gergely, 2009). For example, for most Drosophila cells and for several vertebrate cell lines it was described that additional centrosomes cluster in two distinct spindle poles leading to a bipolar spindle (Basto et al., 2008; reviewed in Nigg & Raff, 2009). Even inactivation of unclustered centrosomes was observed in Drosophila cells (Basto et al., 2008). However, in the same study Basto et al. also showed that larvae brain cells displaying centrosome amplification (by SAK (Sak kinase) overexpression) cause metastatic tumors if implanted in adult wild-type flies. The authors explain this contradictory results with the fact that in slow dividing somatic cells of adult Drosophila several mechanisms work synergistically to prevent the formation of multipolar spindles, while in neuroblasts distorted asymmetric cell division may lead to an increased cell population (Basto et al., 2008). Conversely, other studies describe centrosome supernumerary rather as a result than cause of disrupted cytokinesis and erroneous mitotic progression (Meraldi et al., 2002), which leads to a tetraploid state in P53 (p53 tumor suppressor) depleted cells. Meraldi et al. explicitly state that this is not excluding that centrosome anomalies, once apparent, might cause chromosome missegregation leading to aneuploidy. In 2009, Ganem et al. reported that centrosome amplification can result in chromosome missegregation even in case of bipolar division ensured by centrosome clustering. They observed that multipolar spindles during the intermediate phase lead to accumulation of merotelic (and syntelic) microtubule-kinetochore attachment errors, before bipolar spindles are formed by clustering. These errors caused chromosome missegregation (Ganem et al., 2009).

A correlation between centrosome defects and tumor progression, grading, and prognosis has been described for many soft tissue and solid cancer types (extensively reviewed in Chan, 2011; reviewed in Godinho & Pellman, 2014). For instance, the
dysfunction of BRCA1-BARD1 (breast cancer 1 - BRCA1 associated RING domain 1), which functions as an E3 ubiquitin ligase for γ-tubulin, favors the development of supernumerary centrosomes (Starita et al., 2004). Further, Aurora-A overexpression, a kinase which recruits γ-tubulin at the centrosome base (Hannak et al., 2001), and centrosome amplification were shown to be early events (before malignancy) in the development of rat mammary tumors (Goepfert et al., 2002).

A recent study by Gondinho et al. revealed a mechanistic link between centrosome amplification, increased microtubule nucleation and disturbed cell-cell adhesion via the Rac1 (RAS-related C3 botulinum substrate 1) pathway favoring tumor invasiveness (Godinho et al., 2014). Lingle et al. on the other hand showed that centrosome number as well as centrosome size are correlated with chromosome instability in primary breast tumor cells (Lingle et al., 2002). In summary, although the role of centrosome defects in tumor progression was questioned, during the last decade strong evidence emerged indicating a central role of centrosome defects in tumor progression supporting Boveri’s century old hypothesis.

Autosomal recessive primary microcephaly (MCPH) is characterized by a decreased brain size, while maintaining intact brain architecture. The postnatal head circumference of affected individuals is smaller than for comparable unaffected individuals. A further common symptom is a reduction of intellectual abilities (reviewed in C G Woods, Bond, & Enard, 2005; C Geoffrey Woods, 2004). To date 12 loci causing microcephaly have been identified namely MICROCEPHALIN, WDR62 (WD repeat domain 62), CDK5RAP2, CASC5 (cancer susceptibility candidate 5), ASPM (abnormal spindle microtubule assembly), CPAP (centrosomal P4.1-associated protein), STIL (SCL/TAL1 interrupting locus), CEP135 (centrosomal protein 135 kDa), CEP152, ZNF335 (zinc finger protein 335), PCH1 (pontocerebellar hypoplasia 1), and CDK6 (cyclin-dependent kinase 6), whereby most of these proteins are associated with the centrosome during certain stages of the cell cycle. Mutations often lead to malfunctioning truncated versions of the proteins (reviewed in Barbelanne & Tsang, 2014; C G Woods et al., 2005). Since in general MCPH genes are highly active in neuroprogenitor cells, the proposed mechanism for the development of MCPH assumes a disturb symmetric cell division of neuroprogenitor cells. This would result in a reduced number of neuroprogenitor cells, which in turn lowers the number of generated precursor cells during asymmetric cell division (reviewed in Barbelanne & Tsang, 2014). Furthermore, it was recently shown in mouse models that centrosome amplification can result in microcephaly (Marthiens et al., 2013).
Disruption of the centriole integrity has severe effects on the cilia and flagella for which they serve as basal bodies. Those cilia defects cause a variety of ciliopathies (reviewed in Bettencourt-Dias & Hildebrandt, 2011). For the disruption of motile cilia the primary ciliary dyskinesia is a classical example. It leads, among others, to sinusitis and bronchitis due to a lack in mucus movement by the cilia and to infertility due to immobile sperm (reviewed in Horani, Ferkol, Dutcher, & Brody, 2015). Abnormalities in body symmetry are often observed when motile cilia cannot establish the necessary directional flow during embryogenesis for symmetry determination (reviewed in Bettencourt-Dias & Hildebrandt, 2011).

1.1.3. Centrosome/Centriole duplication cycle

In order to fulfill its role as component of the bipolar spindle apparatus the centrosome follows its own tightly controlled duplication cycle along the cell cycle. Both cycles are coupled by several key regulators, but can be uncoupled by experimental measures (reviewed in Fu, Hagan, & Glover, 2015).

During the transition from G1 to S phase centrosome duplication is initiated, starting with the formation of new daughter centrioles at the walls of the two apparent centrioles. Being coupled to the DNA replication the centrosome/centriole duplication is controlled by CDK2 (cyclin-dependent kinase 2)/cyclin E. However, it is possible to uncouple the two processes, e.g. via constitutive activation of CDK2/cyclin E (Mussman et al., 2000). Cells with impaired DNA replication, either by S phase arrest or DNA polymerase inhibition, display an overamplification of centrosomes, which can be reversed by CDK5 (cyclin-dependent kinase 5)/cyclin E inhibition (reviewed in Fu et al., 2015; E H Hinchcliffe, Li, Thompson, Maller, & Sluder, 1999; Matsumoto, Hayashi, & Nishida, 1999; P Meraldi, Lukas, Fry, Bartek, & Nigg, 1999; Raff & Glover, 1988). The CDK (cyclin dependent kinase) inhibitory proteins P21 (cyclin-dependent kinase inhibitor 1A) and P27 (cyclin-dependent kinase inhibitor 1B) derivatives have been known to prevent CDK based centrosome duplication (Lacey et al., 1999).

Furthermore, various interaction partners and substrates of CDK's and cyclines have a regulatory function during these early stages of centrosome duplication. NPM/B23 (nucleophosmin) phosphorylation is promoted by CDK2/cyclin E, which causes its dissociation from the unduplicated centrosome, where it has an inhibitory effect on duplication (Okuda et al., 2000; Tokuyama et al., 2001). Cyclin A and E mediate the
localization of MCM5 (minichromosome maintenance complex component 5) to the centrosome. The DNA replication licensing factor was shown to block centrosome duplication in S phase arrested CHO cells (Chinese hamster ovary cells) (Ferguson et al., 2010; Ferguson and Maller, 2008). Similar, cyclin A dependent localization of Orc1 (origin recognition complex subunit 1) to the centrosome was shown to prevent cyclin E mediated duplication (Hemerly et al., 2009). Interestingly, a link between the CDK2/Cyclin E regulation of centrosome duplication with p53 has been suggest by studies showing that cyclin E overexpression in p53 deficient cell lines results in overamplification of centrosomes (Tarapore and Fukasawa, 2002).

After the start of centrosome duplication at the G1 to S phase transition centriole duplication is initiated. The polo like kinase 4 (Plk4) lies at the start of the assembly of the daughter centrioles in human and *Drosophila* (reviewed in Gönczy, 2012), while in *C. elegans* the distant ortholog Zyg-1 (zygote defective protein 1) initiates centriole assembly (O’Connell et al., 2001; Slevin et al., 2012). In human/fly cells Plk4 is recruited to the centrosome by the PCM protein CEP152/Asterless, which interacts via its N-terminal domain with the cryptic polo box of Plk4 (Cizmecioglu et al., 2010; Dzhindzhev et al., 2010; Hatch et al., 2010) and shares this task with CEP192, on which CEP152 also mainly depends for centrosome localization (Kim et al., 2013; Sonnen et al., 2013). In *C. elegans* Zyg-1 is recruited to the site of centriole assembly by Spd-2 the ortholog of CEP192 (Pelletier et al., 2006).

Both Plk4 and Zyg-1 are under a complex regulation. For instance, Plk4 is activated via phosphorylation by SAPKKK(stress-activated protein kinase kinase kinases) during early stages of stress response, while it is downregulated by p53 during late stages of stress response (Nakamura et al., 2013).

During cell cycle progression the level of Plk4 is regulated by the E3 ubiquitin ligase complex SCFlmb/βTrCP (SKP1-cullin-F-box, supernumerary limbs/beta-transducin repeat containing E3 ubiquitin protein ligase) via proteasomal degradation. Plk4 will dimerize with its C-terminal coiled-coil domain (Habedanck et al., 2005; Leung et al., 2002) allowing trans autophosphorylation, which mediates binding by SCF (Cunha-Ferreira et al., 2009; Guderian et al., 2010; Rusan and Rogers, 2009). This inhibitory autophosphorylation is counteracted by the phosphatase PP2A (protein phosphatase 2A), which was shown in *Drosophila* to dephosphorylate Plk4 and thereby preventing the degradation. Similar Zyg-1 is known to be degraded by homologs of SCFlmb/βTrCP, Lin23 (F-box/WD repeat-containing protein lin-23) and SEL-10 (suppressor/enhancer of lin-12 10) (Peel et al., 2012).
In *C. elegans* Zyg-1 binds to SAS-6 (spindle assembly abnormal 6) and SAS-5 (spindle assembly abnormal 5) recruits them to the centriole assembly site independent of its kinase activity (Lettman et al., 2013). SAS-6 and SAS-5 are recruited as a complex to the site of centriole assembly. Whereas SAS-6 continues to reside at the emerging daughter centriole and forms the central tube, SAS-5 shuttles between the cytosol and the daughter centriole (Delattre et al., 2004). Although there are different perceptions on how PP2A contributes to the recruiting process of SAS-6 and SAS-5, either by removing an inhibitory phosphorylation (Kitagawa et al., 2011a) or by protecting Zyg-1 and SAS-5 against degradation (Song et al., 2011), it is clear that PP2A is crucial for the recruiting of SAS-5 to the centriole (reviewed in Megraw, 2011).

In *Drosophila* Plk4, *C. elegans* ortholog of Zyg-1, phosphorylates the SAS-5 ortholog Ana2 (anastral spindle 2) in a conserved motif, which can thereby bind SAS-6 and recruit it to the centriole assembly site (Dzhindzhev et al., 2014). Here, instead of forming a tube-like structure as in *C. elegans*, SAS-6 forms a 9-fold symmetric cartwheel-like structure with outwards protruding spokes (Kitagawa et al., 2011c; van Breugel et al., 2014, 2011). The human SAS-5 ortholog STIL is equally phosphorylated by human Plk4 (Kratz et al., 2015) which triggers SAS-6 recruitment. Depletion of STIL was shown to prevent centriole duplication (Vulprecht et al., 2012). Furthermore, it was show that phosphorylated STIL binds human SAS-6 and localizes at the centriole (Ohta et al., 2014). Interestingly, human SAS-6 only resides transiently at the centrioles (Strnad et al., 2007) and Fong et al. speculated that it is first recruited to the proximal end of the mother centriole during the S phase before being repositioned in a Plk4 dependent manner to the site of the emerging procentriole (Fong et al., 2014). During the subsequent formation of the centriole wall microtubule multiplet the A-microtubules are assumed to be nucleated first by a proximal γ-TuRC complex and serve as scaffold for the assembly of the B- and C-tubule (Guichard et al., 2010).

After the recruitment of SAS-5 and SAS-6, SAS-4 (spindle assembly abnormal 4) is recruited to the site of the new daughter centriole. SAS-4 as well as its human ortholog CPAP and its fly ortholog Ana2 were shown to be responsible for the elongation of the centriolar microtubules during the G2 phase. Further factors involved in centriole elongation and formation of the outer centriole wall are CEP120 and SPICE1 (spindle and centriole associated protein1), which interact with CPAP (Comartin et al., 2013; Guichard et al., 2010; Mahjoub et al., 2010). The centriolar protein CP110 (centriolar coiled-coil protein 110 kDa) on the other hand is recruited by CEP97 (Spektor et al., 2007) to the
distal end of the growing daughter centriole (Fu and Glover, 2012; Kleylein-Sohn et al., 2007) and prevents overelongation (Schmidt et al., 2009) and thereby acts antagonistically to CPAP.

At the onset of mitosis the disjunction of the newly formed pairs of centrioles takes place. During this process the C-Nap1 linker protein between the two old centrioles is phosphorylated by Nek2A (NIMA related kinase 2) and displaced from the centrosome (Mardin et al., 2010). The Nek2A levels are cell cycle dependent and reach a maximum at late S and G2 phase, whereas the critical threshold for disjunction was suggested to be reached during the prophase (Hayes et al., 2006). The disjoint centriole pairs migrate to the spindle poles supported by the kinesin motor protein Eg5 (Kindle Spindle Protein/KSP/KIF11/kinesin-5) (reviewed in Agircan, Schiebel, & Mardin, 2014).

Before the onset of mitosis the maturation of the now two centrosomes continues with accumulation of PCM around the mother centriole. Using a genome wide RNAi screen Dobbelare et al. identified a set of proteins required for PCM accumulation (Dobbelaeere et al., 2008). In human and Drosophila cells, centrosome maturation is mediated by Plk1, which is required for the rapid growth of the PCM around the mother centriole (reviewed in Glover, 2005). Phosphorylation by Plk1 allows for the recruitment of the PCM proteins Spd-2/CEP192, pericentrin, CNN/CDK5RAP2 and NEDD1 (Haren et al., 2009). For drosophila it was shown that Plk1 resides close to the centriole and was suggested to cause a radial decreasing phosphorylation gradient across the PCM.

Initially CNN (Drosophila ortholog of CDK5RAP2) accumulates close to the centriole walls, before it spreads across the PCM and extends the PCM scaffold (Conduit et al., 2010). Human Plk1 was reported to phosphorylate the important PCM scaffold protein PCNT (pericentrin) at the start of centrosome maturation (Lee and Rhee, 2011), which was proposed to result in the recruitment of additional PCM proteins. Interestingly, it was further found that the localization of Plk1 in the vicinity of the centriole wall depends on Spd-2 (Decker et al., 2011).

Further proteins in drosophila, which support centrosome maturation at G2/M transition are SAS-4 which forms the link between the centriole and the PCM (Dzhindzhev et al., 2010) and its interaction partner Asl, which is responsible for the recruitment of PCM proteins to the mother centriole such as CNN (Conduit et al., 2010; Varmark et al., 2007). Additionally, SAS-4 forms complexes with the PCM proteins CNN and Dplp (pericentrin-like protein (Drosophila)) (Dzhindzhev et al., 2010; Gopalakrishnan et al., 2011). Overall,
accumulation of PCM proteins leads to recruitment of γ-tubulin (reviewed in Fu et al., 2015).

After centrosome maturation enables the centrosome to serve its function as microtubule nucleating and organizing spindle pole, the cell progresses through mitosis. At the M to G1 transition the centrosome finalizes its duplication cycle by disengagement of the newly formed daughter centriole from the side wall of the mother centriole. Centriole disengagement is an important licensing step for centriole duplication (Tsou and Stearns, 2006). Separase and Plk1 are important factors promoting the disengagement (Schöckel et al., 2011; Tsou et al., 2009). While Plk1 seems to support disengagement before anaphase, separase seems to be active at the later stages of mitosis (Tsou et al., 2009). The separase is negatively controlled by securin and cyclin B (Gorr et al., 2005). PP2A phosphatase keeps securin dephosphorylated and thereby prevents its phosphorylation-dependent polyubiquitination by APC/C (anaphase-promoting complex/cyclosome E3 ubiquitin ligase) and the subsequent proteasomal degradation (Hellmuth et al., 2014). Hence, centriole disengagement depends on the activity of APC/C-Cdh1 (APC/C -activator protein CDH1 complex), which forms a pathway independent of the Plk1 mediated disengagement (Hatano and Sluder, 2012).

However, what the actual substrate of separase is and what physically links the procentriole to the wall of the mother centriole remains elusive. Cohesin, the protein connecting the sister chromatids, was suggested as the linking agent after experiments in HeLa (Henrietta Lacks) cells with non-cleavable cohesin were conducted (Schöckel et al., 2011). Nevertheless, other studies showed that cohesin cleavage is not sufficient for centriole disengagement (Oliveira and Nasmyth, 2013; Tsou et al., 2009). Recently Pagan et al. identified the PCM proteins CEP215 (CDK5RAP2) and pericentrin as promising candidates for the centriole engagement mechanism (Pagan et al., 2014). They described two pools of CEP215, one located at the proximal end of the centriole interacting with pericentrin and one peripheral pool interacting with Plk1. They could show that Plk1-and β-TrCP-mediated degradation of CEP68 during prometaphase is important for separation of the centrioles after disengagement, hence causing the release of the peripheral CEP215. Furthermore, they showed that separase dependent cleavage and subsequent degradation of pericentrin at the proximal end of the centriole, which frees its interaction partner CEP215, facilitates disengagement. Pagan et al. therefore proposed that a PCM remodeling is supporting centriole disengagement (Pagan et al., 2014). The importance of PCNT-B (pericentrin) cleavage and its dependency on separase had already been
established by previous studies (Lee and Rhee, 2012; Matsuo et al., 2012). This supports a model proposing that a PCM scaffold cage is sterically preventing disengagement, rather than a set of proteins acting as direct physical linkers (although both hypotheses do not mutually exclude each other *per se*) (Cabral et al., 2013). After the disengagement at the end of mitosis the centrioles are ready for the new cycle of duplication.
1.2. Centriole architecture

As outlined above during the S and the G2 phase the actually assembly of new procentrioles takes place. The two core components of the centriole are the cartwheel or in *C. elegans* the central tube and the microtubule centriole wall. The wall consists of nine microtubule blades with up to three microtubules per blade. After the general structure and the duplication cycle of the centrosome were introduced in the preceding paragraphs, the following ones will focus on the components of the centriolar cartwheel and microtubule centriole wall, thereby introducing coiled-coil proteins and microtubules before discussing the two important proteins SAS-6 and CEP135 in detail.

1.2.1. Cartwheel build up

The cartwheel was first described by Gibbons and Grunsbone in 1960 (Gibbons and Grimstone, 1960). It was shown by electron microscopy studies that it consists of an inner hub with nine spokes radiating outwards to which an A-microtubule belonging to a microtubule multiplet is attached via an electron dense pinhead region (reviewed in Hirono, 2014) (Figure 1.3.A). Cryo-electron tomography studies on purified *Trichonympha* centrioles showed that, in the context of a centriole, several cartwheels are stacked on top of each other (Guichard et al., 2013) (Figure 1.3.). The number of stacked cartwheels depends on the organism and the cell cycle stage. For instance, mammalian centrioles only display cartwheels in the emerging procentriole during centriole duplication (reviewed in Alvey, 1986; Strnad & Gönczy, 2008). In *Chlamydomonas* the cartwheel persists throughout the whole cell cycle. Nevertheless, the number of cartwheels in a stack changes from 7-10 in an emerging procentriole to 2-4 in mature centrioles in species such as *Chlamydomonas* and *Spermatozopsis* (Beech and Melkonian, 1993; Geimer and Melkonian, 2004; Lechtreck and Grunow, 1999; O’Toole and Dutcher, 2014). In *Chlamydomonas* the cartwheel stack normally occupies the proximal 100 nm of a 400-450 nm long centriole. Cryo-electron tomography further revealed that in case of the unusually long *Trichonympha* cartwheel stacks the spokes of two neighboring cartwheels are connected roughly 20 nm outwards along the 50 nm long spokes from the cartwheel hub, which has a diameter of ~ 22 nm and a stack periodicity of 8.5 nm (Guichard et al., 2013)(Figure 1.3.B).
Similar connecting densities between the spokes have been suggested for *Chlamydomonas* (Hirono, 2014). It was hypothesized that the 9-fold symmetric cartwheel imposes the 9-fold symmetry onto the centriole (Beisson and Wright, 2003; Cavalier-Smith, 1974). However, also contrary results have been published. In *Paramecium* the cartwheel seems to appear only after the centriolar microtubules (Dippell, 1968). *C. elegans* on the other hand seem to have no cartwheel, but rather a central tube that is located in the lumen of the centriole (Pelletier et al., 2006).
Coiled-coil proteins

Many scaffold proteins in the centriole harbor coiled-coil domains of various length. They have the benefit of providing strong oligomerization and protein-protein interaction domains, while at the same time spanning considerable distances, which makes them ideal for building up large multimeric structures.

The first to predict the coiled-coil arrangement of α-helices were Crick, Pauling and Corey in the early 50’s (Crick, 1953, 1952; Pauling and Corey, 1953). In general a coiled-coil consists of right handed alpha helices coiling around each other, whereby the formed coiled-coil could be a back folded monomeric structure or up to pentameric or even heptameric oligomers in cases of designed coiled-coils (Liu et al., 2006; Malashkevich, Kammerer, Efimov, Schulthess, & Engel, 1996; reviewed in Parry, Fraser, & Squire, 2008). Coiled-coils can be of parallel or antiparallel conformation.

A coiled-coil is normally parameterized by a set of parameters first introduced by Crick. The pitch (rise per superhelical turn), the pitch angle, pairwise helix crossing angle, the superhelix radius and its phase shift, and the α-helix radius and its phase shift (Crick, 1952; Harbury et al., 1993; Offer et al., 2002; Offer and Sessions, 1995). The sequence of residues in coiled-coils follows a characteristic heptad repeat pattern where the different positions are labeled with “a” to “g” (Figure 1.4.A). The important core residues forming the hydrophobic core reside in the a and d positions. In over 70% of all cases those residues are hydrophobic such as leucine, valine and isoleucine (Conway and Parry, 1990). The a and d position residues form a left-handed, hydrophobic helical stretch along the axis of the right-handed α-helices, packing in a knobs into holes fashion (reviewed in Lupas & Gruber, 2005; Walshaw & Woolfson, 2001)(Figure 1.4.B). The residues in the e and g positions are flanking the hydrophobic core, while the c, b, and f positions are exposed towards the solvent (Figure 1.4.A). Ionic interactions between the residues in the e and g positions often contribute to the stability of the coiled-coil (reviewed in Parry et al., 2008).

The α-helices participating in a coiled-coil display a rise of 3.5 amino acids per turn instead of the usual 3.6-3.64, which allows for equivalent positions every 7 amino acids. This leads to a 3-4-3-4 pattern of residue spacing between the hydrophobic core residues in the a and d positions (reviewed in Lupas & Gruber, 2005).

Three different forms of discontinuities disrupting this pattern are known to appear regularly in coiled-coils: 1) the stammer (insertion of three residues), 2) the stutter (insertion of four residues), and 3) the skip (insertion or deletion of one residue). The
stammer is causing a 3-4-3-3-4 pattern and is assumed to form a tighter coiling, while the stutter is creating a 3-4-4-3-4 spacing pattern and was shown to lead to unwinding (Brown, Cohen, & Parry, 1996; reviewed in Parry et al., 2008; Strelkov & Burkhard, 2002). The knobs into holes packing is locally transformed to a knobs to knobs packing in case for stammers and stutters. Discontinuities caused by a skip are compensated in a coiled-coil by various mechanisms such as formation of a local π-helix conformation or introduction of kinks (reviewed in Lupas & Gruber, 2005).

The configuration of the coiled-coil and the number of the participating strands is highly dependent on the type of residues occupying the heptad positions. In a dimeric parallel coiled-coil the vector of the Ca-Cβ bond in an a position is unidirectional with the peptide bond and therefore the adjacent a position residues of the two partner strands are more distant from each other than residues in d positions. To achieve a tight packing, Cβ branched hydrophobic amino acids (e.g., Val, Ile or Thr) are preferred in a positions in order to fill the cavity. For the d positions the vector of the Ca-Cβ bond is not parallel to the peptide bond but rather perpendicular, which results in a smaller gap between d position residues in partner strands. In d positions Cβ unbranched residues such as Leu and Ala are preferred (Harbury et al., 1993; reviewed in Lupas & Gruber, 2005). In 4-stranded coiled-coils the positional preferences are reversed, while 3-stranded coiled-coils display a Ca-Cβ vector angle that is intermediate. Instead of pure a or d position pairs as in parallel coiled-coils, anti parallel coiled-coils pack via mixed a and d position pairing (Harbury et al., 1993).

Studies on the leucine zipper protein GCN4 (amino acid starvation-responsive transcription factor GCN4) showed that the number of participating strands can be artificially tuned, depending on the residues residing in the a and d position. However, these findings from single cases cannot easily be generalized (Campbell and Lumb, 2002; Gonzalez et al., 1996; Harbury et al., 1993, 1994). Further theoretical studies based on molecular dynamics simulations have shown that also the orientation of the strands depends on residue types at key positions. Trp at a core position as well as Arg and Lys residues in the a and d position, were they interact with residues in the e and g position, favor anti parallel conformations. In general, salt-bridge networks were found to be more prominent in antiparallel coiled-coils (Ramos and Lazaridis, 2011).

The typical knobs into holes interaction pattern seems to be required but not sufficient for the assembly of a coiled-coil. For several coiled-coil proteins so called trigger sequences have been identified. Without these sequences serving as nucleation sites the
coiled-coil was not formed (Kammerer et al., 1998; Steinmetz et al., 1998).

Despite being of comparably simple architecture coiled-coils serve many functions inside the cell. They are often used to span long distances, e.g., to act as stalks in the motor proteins kinesin, dynein and myosin. They act as scaffolds, e.g., in the intermediate filament protein α-keratin and the ability of coiled-coils to produce stable oligomers is often used to drive oligomerization of macromolecular complexes (reviewed in Burkhard, Stetefeld, & Strelkov, 2001). Examples for this functionality can be observed in the context of the centriole. Many centriolar proteins contain coiled-coil domains (Carvalho-Santos et al., 2010; Kuhn et al., 2014). They often span large distances as scaffolding support for other proteins to interact with (e.g., the SAS-6 stalk (Qiao et al., 2012)) or facilitate oligomerization to build up larger entities (e.g., SAS-5 (Rogala et al., 2015)). Due to their versatility yet simplicity in build up, coiled-coil domains are important in many of the centrosomal proteins.

Figure 1.4. The coiled-coil heptad positions.
A) Coiled-coil heptad position scheme showing the heptad positions in a parallel dimeric coiled-coil. (Based on Perry et al., 2008)
B) Parallel dimeric coiled-coil section of tropomyosin (PDB code: 1IC2 chain a and b) showing the knobs into holes packing. Heptad a positions: green; heptad d positions: orange. The coiled-coil register was assigned manually.
Bld12p/SAS-6 and its interaction partners

The cartwheel protein SAS-6 (spindle assembly abnormal protein 6) and its Chlamydomonas ortholog Bld12p (basal body protein 12) are well conserved in centriole bearing organisms (Carvalho-Santos et al., 2010). This protein family was first described for C. elegans and H. sapiens and was shown to be required for centriole duplication (Dammermann et al., 2004; Leidel et al., 2005). Immunoelectron microscopy studies showed that Bld12p localizes to the central parts of the cartwheel (Nakazawa et al., 2007). Similar localization to the central cartwheel component was observed for Paramecium and Tetrahymena SAS-6 (Jerka-Dziadosz et al., 2010; Kilburn et al., 2007). Furthermore, Chlamydomonas Bld12p null mutant strains lacked the central components of the cartwheel and displayed mainly fragmented centrioles (Nakazawa et al., 2007). Of the remaining circular centrioles 70% had nine microtubule triplets but were missing the central cartwheel part (reviewed in Hirono, 2014). SAS-6 depletion in Paramecium via RNAi resulted in basal bodies lacking the cartwheel hub and a decreased basal body length and reduced symmetry (Jerka-Dziadosz et al., 2010). A similar phenotype, including lack of centrioles and reduction of centriolar length, was observed upon depletion of SAS-6 in Drosophila and in a SAS-6 null mutant strain of Drosophila cells (Rodrigues-Martins et al., 2007). In C. elegans embryos the assembly of the central tube failed after SAS-6 depletion via RNAi (Pelletier et al., 2006).

Overexpression of SAS-6 in Drosophila on the other hand was reported to lead to the formation of tube-like structures (Rodrigues-Martins et al., 2007). It was suggested that SAS-6 is important for establishing the 9-fold symmetry of the centriole in Chlamydomonas (Nakazawa et al., 2007). How SAS-6 contributes to the build up of the cartwheel structure became clear when the crystal structure of SAS-6/Bld12p from C. reinhardtii (crSAS-6/Bld12p), C. elegans (ceSAS-6), D. rerio (drSAS-6), L. major (ljSAS-6) and lately D. melanogaster (dmSAS-6) was solved in 2011, 2014 and 2015 (Cottee et al., 2015; Hilbert et al., 2013; Kitagawa et al., 2011c; van Breugel et al., 2014, 2011). For all organisms the N-terminus was found to constitute a head domain displaying a 7-stranded β-barrel fold topped by a helix turn helix motif (Figure 1.5.A ). This domain has the ability to form homodimers. The dimerization interface of this N-terminal domain consist of the β6-β7 loop containing a hydrophobic residue protruding into a hydrophobic groove created by the helix-turn-helix motif on the adjacent SAS-6 N-terminal head domain. This hydrophobic residue is either F145 for C. reinhardtii, I154 for C. elegans, F131 for D. rerio, F143 for D.
*melanogaster* and F257 for *L. major*. The K\(_D\) for the head domain dimerization is 60 \(\mu\)M (*C. reinhardtii*), 110 \(\mu\)M (*C. elegans*), 90 \(\mu\)M (*D. rerio*), and \(\sim 600\) \(\mu\)M (*L. major*); respectively (Kitagawa et al., 2011c; van Breugel et al., 2014, 2011). Mutation of the protruding hydrophobic residue confirmed its importance in all organisms. The mutation of F131E in human SAS-6, the residue in the position equivalent to I154 in *C. elegans*, likewise impaired N-N head interaction. This was indicated by the inability of exogenous F131E mutants to rescue centriole formation upon depletion of the endogenous SAS-6 in human cells (Kitagawa et al., 2011c). The respective mutations I54E in *C. elegans*, F145E in *C. reinhardtii*, F131D in *D. rerio*, F143D for *D. melanogaster*, and F257E in *L. major* had a comparable effect on N-N head dimerization.

Structural analysis of SAS-6 further revealed that a potential coiled-coil domain was located carboxy-terminal to the N-terminus head domain. CD measurements and studies of disulfide bridge formation ability confirmed the predicted parallel coiled-coil conformation in *C. elegans* (Kitagawa et al., 2011c).

Different groups succeeded in solving the crystal structures of the N-terminal head domains with parts of the C-terminal coiled-coil spokes after disrupting the N-terminal head domain homodimerization (NN dimerization) by mutations. For *C. reinhardtii*, *D. rerio* and *L. major* the crystal structures confirmed the dimerization of two SAS-6 moieties via their C-terminal coiled-coil tail. The extrapolation and combination of the structures of the NN head dimer and the coiled-coil mediated dimer led to the conclusion that SAS-6 is able to form 9-fold symmetric rings. In that model SAS-6 coiled-coil homodimers interact via their N-terminal head domains to generate a hub-like 9-fold symmetric ring with coiled-coil spokes radiating outwards (Figure 1.5.BC) (Kitagawa et al., 2011c; van Breugel et al., 2014, 2011).

This model was further supported by electron microscopy studies showing the ability of SAS-6 to indeed generate 9-fold symmetric rings with a hub diameter of 22 +/- 2 nm or V-shaped dimer of dimers with angles allowing for ring formation (Kitagawa et al., 2011c). Recently, Van Breugel et al. succeeded in crystallizing a SAS-6 construct of *L. major* bearing both homodimerization domains. This construct crystallized as a 9-fold symmetric ring and showed stacks of rings (van Breugel et al., 2014). This delivered the final piece of evidence that SAS-6 has the ability to generate a 9-fold symmetric ring, reminiscent in its dimension and appearance of the centriolar cartwheel. The current understanding is that SAS-6 is the major constituent of the cartwheel hub and of large parts of the cartwheel spokes (reviewed in Hirono, 2014).
Interestingly, the structural analysis of *C. elegans* SAS-6 delivered a possible explanation for the existence of a central tube rather than a cartwheel in the proximal lumen of the *C. elegans* centriole. The structures of two different constructs of the ceSAS-6 N-terminal head domain with coiled-coil stalk were solved. They were engineered to have either a I154G point mutation or a Δ151-156 deletion to prevent dimerization of the N-terminal head domains and a deletion of the N103- P130 loop to improve crystal packing (Hilbert et al., 2013). In both cases the N-terminal head domain and the C-terminal coiled-
coil stalk were comparable in structure to those solved for *C. reinhardtii* and *D. rerio*. However, the orientation of the two domains towards each other differed by approximately 38° compared to previous SAS-6 structures from different organisms. Subsequent modeling and molecular dynamics simulation (MD) revealed the possibility that ceSAS6 could form a 9-fold symmetric spiral. Negative stain EM studies with a S123E/I154W mutant to stabilize the N-terminal head mediated dimerization revealed spiral-like structures similar in dimensions to the spiral model suggested by MD and crystal structures. Furthermore, double spirals consisting of intertwined single spirals were observed (Hilbert et al., 2013). This spiral formation might explain why in *C. elegans* the centriole assembles rather around a central tube than around a stack of cartwheels. In addition, structural studies by Qiao et al. on the coiled-coil domain of ceSAS-6 led to the suggestion that SAS-6 dimers assemble into antiparallel tetramers in solution and that these tetramers are disrupted by binding of ceSAS-5 (Qiao et al., 2012).

While it seems clear now that SAS-6 is the central structural component of the cartwheel and its spokes, it is still an ongoing debate whether SAS-6 alone is strong enough to build a viable cartwheel and to enforce the 9-fold symmetry. Several binding partners may add to the 9-fold symmetry of the cartwheel or its stability. The presumed orthologs SAS-5 (*C. elegans*), Ana2 (*D. melanogaster*), and STIL (*H. sapiens*), sharing two short sequence motifs STAN (Stil/Ana2 motif) and TIM (truncated in microcephaly) (Arquint et al., 2015; Naomi R Stevens et al., 2010; Vulprecht et al., 2012), are important during cartwheel assembly (Avidor-Reiss and Gopalakrishnan, 2013a). ceSAS-5 is binding with its C-terminal α-helical domain (residue 390-404) to a coiled-coil region of ceSAS-6 (residue 275-288). Isothermal titration calorimetry as well as docking experiments using a crystal structure of the ceSAS-6 coiled-coil domain and the predicted structure of the SAS-5 C-terminal domain suggested that one ceSAS-5 C-terminal domain is binding both strands of the SAS-6 coiled-coil. It was therefore suggested that SAS-5 might crosslink the SAS-6 spokes in the context of the centriole (Qiao et al., 2012).

SAS-5 itself was shown to comprise two oligomerization domains. The so called IMPLICO (*Latin*: to entangle) domain of SAS-5 forms a tight homodimer, while the SAS-5 coiled-coil domain is forming a parallel coiled-coil homotrimer. These two sites allow SAS-5 to assemble into large oligomers, such as tetramers and hexamers, important for centriole formation (Rogala et al., 2015). This shows that SAS-5 has the potential to contribute to the build up of multimeric structures like the cartwheel.

The SAS-5 ortholog Ana2 binds to SAS-6 with its conserved STAN motif and co-
overexpression with SAS-6 results in the assembly of cartwheel-like structures (Naomi R Stevens et al., 2010; Naomi R. Stevens et al., 2010; Tang et al., 2011). Furthermore, Ana2 has the ability to tetramerize via its central coiled-coil domain (Cottee et al., 2015).

The human SAS-5/Ana2 ortholog STIL on the other hand is not directly binding to SAS-6 and is rapidly shuttling between the cytosol and the centriole. However, STIL is binding to CPAP’s G-box motif (Cottee et al., 2013; Hatzopoulos et al., 2013; Kitagawa et al., 2011b; Tang et al., 2011). At the end of the cartwheel-spokes an electron dense area, called the pinhead, is connecting the cartwheel to the centriolar microtubule wall. A potential constituent of the pinhead region and interaction partner of SAS-6 is the important centriolar protein CEP135/Bld10p (basal body protein 10), which will be discussed in the dedicated section below. CPAP binds to this interaction partner of SAS-6 (Lin et al., 2013), and with a microtubule as well as a tubulin binding site (Hsu et al., 2008; Hung et al., 2004), it is reasonably equipped to perform the observed function of elongating centriolar microtubules. Also for SAS-6 itself a microtubule binding domain and a microtubule polymerase activity have been recently described for the unstructured far C-terminus (Gupta et al., 2015). In order to clarify the overall contribution of SAS-6 to the 9-fold symmetry and the centriole assembly, a reliable system for in vitro studies on SAS-6 oligomers is desirable. In order to fulfill its purpose it should assemble into stable SAS-6 rings and be tunable with respect to the symmetry of the ring.
1.2.2. The centriole wall

The major components of the centriole wall are the centriolar microtubule triplets. They are arranged in a 9-fold symmetric array around the long axis of the centriole. Many of the introduced centriolar proteins interact with, or can be considered to be part of the centriole wall. In the following sections the main component of the centriole wall, the microtubules, will be discussed. Furthermore, the potential interaction partner of the centriolar microtubules, CEP135/Bld10p, will be introduced.

Microtubules

Besides actin and intermediary filaments, microtubules are an integral part of the cytoskeleton in eukaryotes. Among other functions they serve as cellular roads for motor proteins to transport cargo or to generate the necessary force during cell division or cilia and flagella movement (reviewed in Nogales, 2000). Microtubules are dynamic, hollow, tubular polymers of about 24 nm in diameter build up by heterodimers of α- and β-tubulin (Kirschner et al., 1974). α- and β-tubulin have a globular fold with a N-terminal nucleotide binding site similar to the Rossmann fold able to bind and hydrolyze GTP (guanosine-5'-triphosphate). At their C-terminus, α- and β-tubulin have an unstructured tail, which is often posttranslationally modified. α- and β-tubulin appear in different isoforms, which are expressed in different tissues and vary slightly in sequence and their posttranslational modifications (reviewed in Nogales, 2015).

The nucleotide binding pocket of α-tubulin (N-site) is blocked by the intra-dimeric interface and harbors a non-exchangeable and non-hydrolyzable GTP. The nucleotide binding pocket of β-tubulin (E-site) on the other hand is accessible and can hydrolyze and exchange its nucleotide in context of the αβ-tubulin heterodimer (reviewed in Nogales, 2015). The majority of microtubules consist of 13 protofilaments, which are formed by inter-dimeric longitudinal contacts between the β-tubulin of one dimer and the α-tubulin of the next dimer (Figure 1.6.AB). Lateral contacts between the protofilaments establish a tube, resembling a 3-start helix with respect to the tubulin monomers (Mandelkow et al., 1986). The lateral contacts are in general homotypic, between α-/α- and β-/β-tubulin, apart from the region were heterotypic α-/β-tubulin contacts appear between two adjacent protofilaments. This region is termed “seam” (Mandelkow et al., 1986). The homotypic lattice configuration is termed B-lattice in contrast to the heterotypic A-lattice. Cryo-electron microscopy experiments using kinesin motor proteins, which foremost bind to β-tubulin
(Song and Mandelkow, 1993), showed that in vitro polymerized and purified flagella microtubules are arranged in a B-lattice (Kikkawa et al., 1994). Even cytosolic microtubules decorated with the kinesin Eg5 in perfused cells showed a clear B-lattice, although specific microtubule interacting proteins in the cell (e.g., EB1 (end binding protein-1)) would favor an A-lattice (McIntosh et al., 2009).

The generated microtubule lattice displays specific properties. The microtubule has a faster growing plus end and a shrinking minus end. In vitro it was shown that both ends can grow, although with different rates (reviewed in Dammermann, Desai, & Oegema, 2003). Cryo-electron microscopy studies showed that plus ends appear to have tapered protofilaments of uneven lengths and display an outward curvature without loss of lateral contacts (Chrétien et al., 1995; Mandelkow et al., 1991). The minus end displays outwards peeling protofilaments with loosened lateral contacts to the next protofilament (Mandelkow et al., 1991). In general, while at the plus end of the microtubule new tubulin dimers with bound GTP are added to the protofilaments, GDP (guanosine-5'-diphosphate) bound tubulin dimers dissociate at the minus end. This effect is described as treadmilling (reviewed in Margolis & Wilson, 1998).

Crystallographic studies of heterodimeric tubulin in complex with agents preventing polymerization, e.g., proteins from the stathmin family, display a curved conformation for the GDP tubulin which was comparable to the curvature of the peeling minus end protofilaments (Gigant et al., 2000; Ravelli et al., 2004; Steinmetz et al., 2000). It was previously assumed that the tubulin conformation would depend on the nucleotide state, but a range of studies showed that free tubulin has a curved conformation regardless whether GTP or GDP is bound (Ayaz et al., 2012; Buey et al., 2006; Nawrotek et al., 2011; Pecqueur et al., 2012; Rice et al., 2008). It was shown that the transition from the straight to the curved conformation is induced upon incorporation into an existing microtubule plus end lattice and that bound GTP increases the likelihood of tubulin incorporation into protofilaments (reviewed in Brouhard and Rice, 2014).

In the microtubule lattice, nucleotide hydrolysis at the E-site is possible, but not nucleotide exchange, so that at the plus end the GTP bound form of tubulin dominates (often referred to as the GTP cap), while towards the minus end hydrolysis of GTP to GDP causes the older tubulin layers to exist in a GDP bound state. The GTP hydrolysis in context of the MT lattice leads to a compression in the longitudinal inter-dimer interface, which resides close to the E-site (Alushin et al., 2014). A recent high resolution cryo-EM structure by Zhang et al. showed the M-loop engaging in lateral contacts via its
hydrophobic residues H283 (α-tubulin) and Y283 (β-tubulin), respectively (Zhang et al., 2015). The aromatic residues fit into a cavity generated by the H2-S3 and the H1'-S loop on the laterally adjacent tubulin.

During certain events termed catastrophe in the dynamic life cycle of the microtubule those lateral contacts are disrupted and microtubule growth is exchanged for rapid shrinking until a rescue event resumes the microtubule growth (reviewed in Brouhard, 2015). Catastrophe was shown to correspond to a multistep process involving an age dependent change of catastrophe likelihood and is more likely for old microtubules (reviewed in Brouhard, 2015; reviewed in Gardner, Zanic, & Howard, 2013; Gell et al., 2010; Odde, Cassimeris, & Buettner, 1995; Odde, Tanaka, Hawkins, & Buettner, 1996). This is probably due to the accumulation of lattice defects (Bowne-Anderson et al., 2013; Gardner et al., 2011) or due to increased tapering (Coombes et al., 2013) in combination with the reduction of the GTP cap (reviewed in Brouhard, 2015).

In context of the cellular environment, microtubules rarely contain free ends. In general the plus end as well as the minus end and the microtubule lattice interact with a wide variety of proteins usually termed MAPS's (Microtubule Associated Proteins) which modulate and guide the dynamic behavior and functionalities of microtubules. Many of those proteins target the growing plus end. Furthermore, specific proteins regulate the microtubule dynamics positively (microtubule stabilizing proteins and polymerases) or negatively (microtubule destabilizing proteins, depolymerases and microtubule severing proteins).

A classical example for a protein which acts as a depolymerase is the stathmin protein family. Stathmins can stabilize and/or induce a curved conformation in α-/β-tubulin (Gigant et al., 2000; Steinmetz et al., 2000) and were shown to have a strong promoting effect on catastrophe (Belmont and Mitchison, 1996). It was long debated whether the microtubule destabilizing effect of stathmin is caused by sequestering of free tubulin or by direct interaction with the microtubule lattice (Cassimeris, 2002). Recent results showed that a direct interaction with the microtubule lattice could be responsible by causing curvature in tubulin moieties at the microtubule tip (Gupta et al., 2013). Analytical and stochastic modeling by Zeitz and Kierfeld derived basic differences in the catastrophe switching behavior of the two models, which could potentially be used to distinguish between the two modes of action experimentally (Zeitz and Kierfeld, 2014). However, it is likely that both mechanisms contribute to the stathmin induced effects on microtubule stability (reviewed in Cassimeris, 2002; K. K. Gupta et al., 2013; Howell, Larsson,
Counteracting the microtubule depolymerases are the microtubule polymerases. One well studied example is the protein XMAP215 (microtubule associated protein 215 kDa) that was initially discovered as a microtubule elongating agent in *Xenopus* extract (Gard and Kirschner, 1987). XMAP215 and its homologs were shown to contain several tubulin binding TOG (tumor overexpressed gene) domains that bind to curved αβ-tubulin (Al-Bassam et al., 2006; Slep and Vale, 2007; Wang and Huffaker, 1997). XMAP215 and its homolog Alp14 (altered polarity 14, from *S. pombe*) processively catalyze microtubule polymerization at the microtubule plus end (Brouhard et al., 2008). Recent structures of the TOG1 and TOG2 domain of XMAP215 bound to tubulin helped to explain its preference for the curved αβ-tubulin. TOG domains bind to a region which is structurally distinct for the straight and curved tubulin conformation (Ayaz et al., 2014, 2012). Furthermore, XMAP215 also comprises a basic microtubule lattice binding region C-terminal to the TOG domains (Widlund et al., 2011). The proposed model of function is that XMAP215 binds to the microtubule lattice via its basic region and to the plus end via some of its TOG domains, while other N-terminal TOG domains protrude in the cytosol. There they may bind to curved αβ-tubulin dimers, thereby increasing the local concentration of tubulin dimers at the plus end (Ayaz et al., 2014).

Another class of MAP proteins generally termed the +TIP proteins targets specifically the plus end or the lattice close to the plus end and tip track the plus microtubule end. There the +TIP proteins form a regulatory network influencing microtubule plus end dynamics (Akhmanova and Steinmetz, 2008).

While many proteins were found to interact with the lattice or the plus end in cells, the minus end was mainly described as being anchored to the microtubule organizing center, i.e., the centrosome (see Chapter 1.1.1) or capped by microtubule nucleating γ-tubulin complexes to be protected from dissociation and shrinking (reviewed in Teixidó-Travesa, Roig, & Lüders, 2012). However, recently a protein family was identified binding at the minus end and modulating its dynamicity. The so called CAMSAP proteins (calmodulin-regulated spectrin-associated protein) bind specifically to the growing minus end. They stabilize it and counteract microtubule depolymerases such as kinesin 13, which would otherwise depolymerize those ends (Goodwin and Vale, 2010). While it was shown that the subtype CAMSAP1 only binds transiently to growing microtubule minus ends and thereby tip tracks the minus end, CAMSAP2 and 3 bind to the microtubule lattice generated by the growth (Hendershott and Vale, 2014; Jiang et al., 2014). In general
CAMSAP's function is to stabilize the growing minus end (Jiang et al., 2014; Tanaka et al., 2012; Yau et al., 2014). Depletion studies confirmed the significance of this stabilization of free microtubule minus ends in different cell types from several organisms (reviewed in Akhmanova & Hoogenraad, 2015; reviewed in Silva & Cassimeris, 2014).

Different proteins carry out posttranslational modifications on tubulin and thereby increase its functional versatility. Nearly in all cases the posttranslational modification is located at the unstructured C-terminal tail creating a tubulin code (reviewed in Verhey & Gaertig, 2007; Wehenkel & Janke, 2014). The known types of modifications include, among others, polyglutamylation, acetylation, phosphorylation, and tyrosination (Janke, 2014). Acetylation is one of the few occurring modifications at the structured, globular domain of tubulin. Mainly α-tubulin K40, oriented towards the microtubule lumen, is modified by the acetylase α-Tat1 (α-tubulin N-acetyltransferase 1) (Akella et al., 2010; Shida et al., 2010). Acetylation was suggested to effect cellular trafficking and the binding of inner microtubule proteins (Dompierre et al., 2007; Linck et al., 2014; Reed et al., 2006). HDAC6 (histone deacetylase 6) and Sirt2 (NAD-dependent deacetylase sirtuin-2) were identified as potential deacetylases (Hubbert et al., 2002; North et al., 2003). Further potential acetylation sites were recently described (Choudhary et al., 2009).

Tyrosination at the C-terminal tail of α-tubulin is achieved by the tubulin tyrosine ligase (TTL) (Arce et al., 1975; Hallak et al., 1977). Due to the structure of the binding interface TTLs act specifically on non-polymerized tubulin (Prota et al., 2013; Szyk et al., 2011). Specific detyrosination of tubulin incorporated in the microtubule lattice by an enzyme that has not been identified to date protects microtubules from depolymerases such as kinesin 13 (Peris et al., 2009; Sirajuddin et al., 2014). A lack of tyrosination on the other hand leads to prenatal death of TTL- knockout mice (Erck et al., 2005), thereby showing the significance of tyrosination as tubulin modification.

Strong polyglutamylation is generally observed for axonemes of cilia and flagella and centriolar microtubules (Bobinnec et al., 1998; Fouquet et al., 1994). It is mediated by tubulin tyrosine ligase-like proteins (TTLL) in an ATP-dependent manner (reviewed in Yu, Garnham, & Roll-Mecak, 2015). They catalyze the addition of glutamate at the γ-carboxyl group of a glutamate in the primary sequence of the C-terminal tubulin tail via an isopeptide bond. Further elongation of the polyglutamate chain is achieved by normal peptide bonds. Deglutamylation is catalyzed by cytosolic carboxy peptidases (CCPs) (Kimura et al., 2010; Rogowski et al., 2010). Studies showed that hyperglutamylation due to missing CCP1 leads to neurodegeneration in mouse models (Rogowski et al., 2010).
Due to the microtubules central role in cellular key processes such as cell division, where they form the spindle fibers and are critical for force generation to separate the chromosomes, they have been associated with many pathogenic conditions such as Alzheimer disease (reviewed in Dubey, Ratnakaran, & Koushika, 2015). A variety of drugs to stabilize or destabilize microtubules has been developed, which are also of great value as research tools. The most important ones today are taxol and its derivatives. Originally extracted from the pacific yew tree, it is one of the widest used chemotherapeutics (reviewed in Jordan & Wilson, 2004) and acts via stabilizing microtubules (Schiff and Horwitz, 1980). Several structural studies by cryo-electron microscopy and crystallography demonstrated that taxol binds in a luminal pocket on β-tubulin close to the M-loop which favors and strengthens the lateral contacts in the microtubule lattice (Nogales et al., 1999, 1998; Xiao et al., 2006). Beside its clinical application taxol is therefore used for in vitro polymerization and stabilization of microtubules (Kumar, 1981). Other drugs, such as vinblastine, disrupt the longitudinal contacts and thereby act as microtubule destabilizing agents (Cormier et al., 2010).
In context of the centriole, polyglutamylated microtubule multiplets form the microtubule wall. The numbers of microtubules participating in a multiplet varies among organisms. While in *C. elegans* embryos each microtubule blade of the centriole consists of only one microtubule and in *D. melanogaster* embryos of two microtubules per blade, in other organisms centrioles have triplet microtubules (named A-, B-, and C-microtubule) forming the centriole wall (reviewed in Winey & O’Toole, 2014). Cryotomography studies by Guichard et al. on *Trichonympha* basal bodies showed that the first microtubule per

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**Figure 1.6. Microtubules**

A) Microtubule section generated from 5.5 Å EM structure (PDB code: 3J6G). Gray: α-tubulin; black: β-tubulin; blue: GTP; cyan: GDP

B) Close up front view and 90° turn of a tubulin dimer from the microtubule lattice in A). Gray: α-tubulin; black: β-tubulin; blue: GTP; cyan: GDP; orange: taxol

C) Cryotomography density map of the *Trichonympha* basal body microtubule triplet (EMD: 2330).
blade (termed A-microtubule), which is attached by the pinhead region to the cartwheel spokes, consist of 13 protofilaments (Figure 1.6.C). The B-microtubule is connected to the A-microtubule and consist of only 10 protofilaments. The C-microtubule in turn is again connected to the B-microtubule and also contains 10 protofilaments (Guichard et al., 2013). Both the B- and the C-microtubules have a crescent-like appearance. For different organisms a linker between the C-microtubule and the A-microtubule of the adjacent microtubule blade has been described. Which proteins constitute this A-C linker remains elusive.

**CEP135/Bld10p**

CEP135/Bld10p (centrosomal protein 135 kDa/ basal body protein 10) was first described in the early 2000’s (Ryu et al., 2000; Ohta et al., 2002). Studies in cells confirmed the functional importance of CEP135/Bld10p, in order to maintain proper centriole architecture and flagella formation, although to various degrees for different model organisms.

The CEP135 ortholog Bld10p was shown to be essential in *Chlamydomonas*, *Tetrahymena* and *Paramecium*. The reported phenotypes range from destabilization of the centriole, loss of centriolar microtubules upon depletion in *Tetrahymena*, over complete loss of centrioles and basal bodies in *Chlamydomonas* if depleted, to functional importance for basal body assembly and SAS-6 maintenance in *Paramecium* (Bayless et al., 2012; Hiraki et al., 2007; Jerka-Dziadosz et al., 2010; Matsuura et al., 2004).

For *Drosophila*, it was consistently shown that, although not essential for centriole duplication, Bld10p depletion leads to shortened centrioles, increased centriole diameters and that Bld10p is implicated in the formation of the central microtubule pair in basal bodies. Bld10p was also shown to have an implication for the asymmetric cell division of neuroblasts (Mottier-pavie & Megraw, 2009; Carvalho-Santos et al., 2012; Roque, Wainman, & Richens, 2012; Singh, Ramdas Nair, & Cabernard, 2014). In the case of vertebrates, however, the reported phenotypes vary. Only weak alterations of centriolar numbers were observed upon CEP135 gene disruption in DT40 chicken cells (chicken bursal lymphoma cell line) (Lalor et al., 2013). Primary fibroblasts derived from patients suffering from microcephaly, caused by a premature termination codon in the human CEP135 gene, were reported to have (to a certain extent) multiple centrosomes or complete loss of centrosomes (Hussain et al., 2012).
Mutations in the human CEP135 in regions thought to be involved in interactions with the cartwheel protein SAS-6 or the centriolar protein CPAP, were shown to block Plk4 induced overduplication or CPAP induced elongation of the centriolar microtubules, respectively. Furthermore, a loss of centriolar microtubules, reduction in centriolar length and an increase in the number of abnormal or monopolar mitotic spindles upon depletion were reported (Lin et al., 2013).

Thus, the majority of the CEP135/Bld10p studies discussed above report a stabilizing function on centrioles and basal bodies by presumably interacting with the centriolar microtubule wall. Localization studies on different organisms confirmed that CEP135 localizes accordingly.

Using super resolution microscopy, Sonnen et al. were able to identify the proximal lumen of the mother centriole as the major localization site of human CEP135 (HsCEP135) whereby only a weak co-localization of CEP135 with the cartwheel protein SAS-6 could be observed from the S/G2 to the G2/M transition at the proximal end of the procentriole (Sonnen et al., 2012). This is in agreement with the results from Kleylein-Sohn et al., who could show by fluorescent light microscopy and with immunolabeling electron microscopy that HsCEP135 localizes to the proximal lumen of the nascent centriole and, to a lesser extent, to the proximal lumen of the growing procentriole (Kleylein-Sohn et al., 2007). Interestingly, besides a binding site to CPAP (50-460), a potential SAS-6 biding site was roughly identified for HsCEP135, despite only weak co-localization (Lin et al., 2013).

Immunolabeling electron microscopy studies on the *C. reinhardtii* CEP135 ortholog Bld10p on the other hand revealed a localization at the tip of the cartwheel spokes and thereby at the proximal end of basal bodies (consisting of the nascent/mother centriole) and pro-basal bodies in the vicinity of Bld12p, the *C. reinhardtii* ortholog of the cartwheel protein SAS-6 (Hiraki et al., 2007; Matsuura et al., 2004). Immunostaining electron microscopy further showed a localization of the Bld10p orthologs of *Tetrahymena* and *Paramecium* to the cartwheel spokes (Bayless et al., 2012; Jerka-Dziadosz et al., 2010).

Although the localization studies reported a difference in localization with respect to the cartwheel protein SAS-6 among different orthologs, all of them were localized in the proximal lumen of the mother centriole or the basal body. This luminal localization at the proximal end would allow for interaction with the centriolar microtubules. Indeed, it was shown for the *Drosophila* and human isoforms that CEP135/Bld10p contains a binding site for microtubules within the first 190 (human) or 163 (*Drosophila*) amino acids (Carvalho-Santos et al., 2012; Lin et al., 2013).
Further identified interaction partners of CEP135/Bld10p are C-Nap1, CPAP, SAS-6 and dynactin p50. C-Nap1 is a protein implicated in the centriole-centriole linkage during interphase and binds with its C-terminal region (1777-2443) to the C-terminal region (648-1141) of human CEP135. C-Nap1 and CEP135 were shown to co-localize during interphase, but not during mitosis (Kim et al., 2008). Furthermore, it was shown that CEP135 RNAi depletion results in reduced levels of C-Nap1 at the centrosome causing centrosome splitting (Kim et al., 2008). Human CPAP contains a CEP135 binding site located between amino acid 895 to 1338 binding to the N-terminal half of human CEP135 50-460. Similarly, Lin et al. also reported that the C-terminal half of human CEP135 (416-1140) is binding to SAS-6 in the amino acid region 307-536 (Lin et al., 2013).

Uetake et al. reported to have identified the p50 subunit of dynactin as a further interaction partner of CEP135 using yeast-two-hybrid assays, immunoprecipitation and immunostaining in CHO cells. Additionally, an interdependence of the CEP135 level and the p50 level for maintaining centriolar localization was described (Uetake et al., 2004). Recently an alternative small splice isoform of CEP135 (249 amino acids in length) with potential regulatory impact on CEP135 full length was described (Dahl et al., 2015). This isoform differs in its C-terminal 16 amino acids from the sequence of the full length isoform.

The current model in the field is that CEP135/Bld10p is residing in the cartwheel pinhead region and is forming the link between the inner cartwheel components like SAS-6 and the microtubule centriole wall (reviewed in Hirono, 2014) presumingly via its microtubule binding site and its SAS-6 binding site (Figure 1.7.). Considering the interspecies differences in localization and the different interaction partners described in the literature, this model seems too simplistic and the variations among different orthologs too large as to assume that the simplified link model can capture the function of CEP135 in all its complexity. For example, while it was shown that Bld10p constitutes parts of the cartwheel spokes-end together with SAS-6/Bld12p (Hiraki et al., 2007), human CEP135 only co-localizes during a short time in the cell cycle with SAS-6 (Sonnen et al., 2012). Further, it is to date unknown how such a large protein as CEP135 (1141 amino acids) is accommodated in the confined cartwheel region.
However, though the model cannot be generalized and is far from complete, it provides a starting point to outline structural investigations of CEP135/Bld10p. Despite the different interaction partners that were identified, the only potential binding partner that was identified independently in two organisms and that, according to the localization studies discussed above, would be constantly in the vicinity of CEP135 throughout the cell cycle are centriolar microtubules. Together with the identified phenotype of destabilized centriolar microtubules in CEP135/Bld10p depleted cells, this implies that the microtubule binding activity assumed in the current model is of biological relevance. It is further the only functional domain known to date that is shared between the human CEP135 full length isoform and the short, regulatory CEP135mini isoform.

Therefore, the verification and localization of the microtubule binding site at the CEP135 N-terminus and its structural exploration will provide a model for the structure of the microtubule binding N-terminus and its mode of action. This model could be extrapolated and used to refine the existing model to derive hypotheses to guide the further exploration of CEP135.
1.3. Aims of the thesis

The centrosome is of central importance for many cellular processes, such as bipolar spindle formation or development of cilia and flagella. The core structure of the centrosome are the centrioles. Like the centrosome they follow their own tightly regulated duplication cycle along the cell cycle. Over the years the combined efforts of many groups helped to uncover the identities and functions of different proteins involved in the centriolar architecture. With the advent of super-resolution microscopy, establishing the temporal-spatial context of many key proteins became feasible. Despite recent advances, the current models are restricted concerning accuracy. They cannot provide an overall coherent image of the centriole. For further improvement, the implementation of high resolution structural information is crucial. In the last 5 years several groups succeeded in contributing structural information for different protein domains involved in centriolar cartwheel formation and centriole elongation.

The centriolar protein SAS-6/Bld12p was revealed to be a major constituent of the cartwheel central hub and spokes (Chapter 1.2.3). Through two dimerization interfaces, SAS-6/Bld12p can assemble into cartwheel-like 9-fold symmetric oligomers. However, to which extent SAS-6 influences the cartwheel and the centriolar symmetry remains to be resolved. Likewise, how the spokes are connected to the centriolar microtubules requires further investigation. CEP135/Bld10p was described to be the link between the centriolar microtubules and SAS-6/Bld12p and is thought to stabilize the centriole wall (Chapter 1.2.6.). Interestingly, human and Drosophila CEP135 were found to contain a microtubule binding site (Carvalho-Santos et al., 2012; Lin et al., 2013). While the co-localization with SAS-6 is different for human and Drosophila CEP135, the microtubule binding site was roughly located in the N-terminal domains of both orthologs. It is likely that further CEP135/Bld10p orthologs contain a microtubule binding site due to their proximity to the centriolar microtubules (Chapter 1.2.6.). Thus, the microtubule binding site is likely a common feature of CEP135/Bld10p proteins, potentially explaining the stabilizing function of CEP135/Bld10p on the centriolar microtubule wall. Therefore, we aimed at providing a full structural description of the human CEP135 N-terminus.

Furthermore, we investigated the role of SAS-6/Bld12p in centriole symmetry determination. Based on the hypothesis that a modification of the binding strength influences the symmetry of the SAS-6/Bld12p cartwheel-like oligomers, we mutated key residues in the dimeric interface of the SAS-6/Bld12p N-terminal domain. The oligomeric
symmetry of the resulting SAS-6/Bld12p mutants was tunable by the set of chosen point mutations. The effect of selected mutants was evaluated in \textit{C. reinhardtii} and in human cells. To further clarify the potential impact of the cartwheel-centriole wall connection, Bld12p mutants were evaluated in \textit{C. reinhardtii} in context of truncated Bld10p.

The specific aims of this thesis regarding the structure-function relationship of the microtubule binding CEP135/Bld10p N-terminus and SAS-6/Bld12p are therefore:

1) Structure determination of the CEP135 N-terminus, including the microtubule binding site. The applied methods are X-ray crystallography for high resolution structure determination and small angle X-ray scattering to determine the low resolution molecular envelopes in solution. Molecular modeling and molecular dynamics simulations are used to generate a complete model of the HsCEP135 N-terminus (in collaboration with J. Obbeneni, Paul Scherrer Institute).

2) Localization of the microtubule binding site of human CEP135 and verification via rational mutations. The applied methods are microtubule pelleting assays (in collaboration with Dr. N. Olieric, Paul Scherrer Institute) to determine the location of the binding site, immunofluorescence-based pelleting assays to evaluate the effects of the mutants (in collaboration with Dr. G. Hatzopoulos, École polytechnique fédérale de Lausanne) and cryo-electron microscopy to test for regular decoration of microtubules by human CEP135 (in collaboration with Prof P. Guichard, University of Geneva).

3) The generation of symmetry tunable SAS-6/Bld12p mutants. Three different strategies are used to design the mutants during a first round of mutations: I) maximization of the hydrophobic contacts; II) refinement of salt bridge networks; and III) introduction of covalent cysteine linkage. A second round of mutations combined the outcome of the first two strategies. A contribution was made to the second round of mutations in close collaboration with Dr. M. Hilbert.

4) Structure determination of the rational designed SAS-6 mutants to support functional investigations on cartwheel symmetry. To validate the structural impact of the mutations, high resolution structures of representative mutants were obtained by X-ray crystallography (in collaboration with Dr. M. Hilbert).
Chapter 2

The N-terminus of human CEP135 folds into a coiled-coil critical for microtubule binding
2.1. Declaration of contribution

The contribution to this project includes leading the project and the design, cloning, expression, biophysical and biochemical evaluation of all constructs. During the early stages this was done in collaboration with Dr. N. Olieric (group of Prof. M. O. Steinmetz, Paul Scherrer Institute) for the initial constructs, later exclusively independent. Further, the contribution includes all bioinformatical evaluations (apart from the modeling of HsCEP135-N and the molecular dynamic simulation, which was done in collaboration with J. Obbeneni, Paul Scherrer Institute) as well as all crystallization and structure determination performed on this project. The SAXS data were acquired and analyzed in collaboration with John Missimer. We are grateful to Edward Lowe for acquiring the SAXS data of the His-HsCEP135-N. The electron microscopy data was collected and analyzed by Dr. P. Guichard (group of Prof. P. Gönczy, École polytechnique fédérale de Lausanne). The fluorescent based pelleting assay was performed by Dr. G. Hatzopoulos (group of Prof. P. Gönczy, École polytechnique fédérale de Lausanne).

Briefly, the contribution by all authors as stated on the final manuscript: S.K., P. Guichard, J.M.O., N.O., G.N.H., M.H., I.S., J.M., P. Gönczy, and M.O.S. designed the experiments. S.K., P. Guichard, J.M.O., N.O., G.N.H., M.H., I.S., and J.M. conducted the experiments. S.K. and M.O.S. wrote the manuscript with input from all authors.
2.2. Manuscript

The paper based on the following manuscript was published in Structure with the following details:

“The human centriolar protein CEP135 contains a two-stranded coiled-coil domain critical for microtubule binding”

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SUMMARY

Centrioles are microtubule-based structures that play fundamental roles notably in cell division and cilium biogenesis. CEP135/Bld10p family members are evolutionarily conserved microtubule binding proteins important for centriole formation, but how they function at a molecular level is not known. Here, we analyzed in detail the microtubule binding activity of human CEP135 (HsCEP135). X-ray crystallography and small-angle X-ray scattering in combination with molecular modeling revealed that the 158 N-terminal residues of HsCEP135 constitutes an autonomous domain, which forms a parallel two-stranded coiled-coil structure. Truncation analyses of this domain identified a 13 amino acid segment spanning residues 96-108, which confers microtubule binding activity in vitro. Within this coiled-coil segment, we identified three lysine residues (K101, K104 and K108) that form a positively charged patch on the surface of the protein and which contribute to microtubule binding. Our results provide the first structural information on CEP135/Bld10p proteins and offer insights into their microtubule binding mechanism.
INTRODUCTION

The centriole and the related basal body are organelles that are essential for the formation of the centrosome, the major microtubule organizing center in animal cells, as well as of the axoneme in cilia and flagella (reviewed in Avidor-Reiss and Gopalakrishnan, 2013b; Bornens, 2012; Gönczy, 2012; Tateishi et al., 2013). Due to their functional importance, alterations in centriole structure or function are linked to severe human diseases, including several forms of ciliopathies and cancers (reviewed in Bettencourt-Dias and Hildebrandt, 2011; Nigg and Raff, 2009). Centrioles are typically constructed around a nine-fold symmetric ‘cartwheel’ structure consisting of a central hub from which nine spokes emanate. An electron dense region called the ‘pinhead’ bridges each of these spokes via one to three microtubules, depending on the organism, which collectively form the microtubule wall of the centriole. Despite important progress in recent years, the mechanisms that govern formation of the remarkable and evolutionarily conserved architecture of centrioles remain elusive, owing in part to a paucity of structural information of participating components.

Several players important for promoting centriole assembly and stability have been identified over the last decade in different organisms (Azimzadeh and Marshall, 2010; Gönczy, 2012; Hirono, 2014; Strnad and Gönczy, 2008). Amongst them, the essential cartwheel protein SAS-6 was one of the first to have been studied in great detail: Structural and biophysical data revealed that the self-assembly properties of SAS-6 establish the central hub and a large part of the spokes of the cartwheel, and are thus key in determining the nine-fold symmetry of centrioles (Hilbert et al., 2013; Kitagawa et al., 2011c; Nakazawa et al., 2007; van Breugel et al., 2014, 2011). Another evolutionarily conserved and important centriolar protein is CEP135/Bld10p (Ohta et al., 2002; Ryu et al., 2000), which plays a critical role for proper centriole formation in the unicellular organisms Chlamydomonas, Paramecium and Tetrahymena. Depletion phenotypes in these organisms range from a complete loss of centrioles in Chlamydomonas to defects in centriole assembly and SAS-6 maintenance in Paramecium, or loss of centriolar microtubules in Tetrahymena (Bayless et al., 2012; Hiraki et al., 2007; Jerka-Dziadosz et al., 2010; Matsuura et al., 2004). In Drosophila, CEP135/Bld10p depletion leads to shortened centrioles and to an increase in centriole diameter (Mottier-Pavie and Megraw, 2009; Roque et al., 2012). Furthermore, Drosophila CEP135/Bld10p is critical for assembly of the central microtubule pair of the sperm axoneme (Carvalho-Santos et al., 2012), and plays an important role for asymmetric cell division of neuroblasts (Singh et al., 2014).
the case of chordates, the reported CEP135/Bld10p depletion phenotypes vary somewhat. In DT40 chicken cells, for example, only weak alterations in centriole numbers were observed upon CEP135/Bld10p gene disruption (Lalor et al., 2013). By contrast, primary fibroblasts derived from patients suffering from microcephaly due to a premature termination codon in the human CEP135/Bld10p gene display aberrations in centrosome numbers (Hussain et al., 2012). Furthermore, siRNA-mediated depletion of CEP135/Bld10p in human cultured cells causes a loss of centriolar microtubules, as well as a reduction in centriole length (Dahl et al., 2015; Lin et al., 2013), reminiscent of the phenotype obtained in Drosophila. Together, these observations suggest that CEP135/Bld10p plays an important role in promoting centriole assembly and stability, although its exact mode of action appears to differ somewhat depending on the species.

Immunofluorescence- and electron microscopy (EM) studies ascertained that human CEP135/Bld10p localizes principally to the proximal lumen of the parental centriole and to a lesser extent to the lumen of the growing procentriole (Kleylein-Sohn et al., 2007; Sonnen et al., 2012). On the other hand, work in Chlamydomonas and Paramecium revealed the presence of CEP135/Bld10p at the cartwheel-microtubule connection in the proximal part of centrioles (Hiraki et al., 2007; Jerka-Dziadosz et al., 2010; Matsuura et al., 2004). Interestingly, a microtubule binding site has been mapped to the N-terminal part of human (segment 1-190) and Drosophila (segment 1-163) CEP135/Bld10p (Carvalho-Santos et al., 2012; Lin et al., 2013). Collectively, these studies suggest that CEP135/Bld10p proteins stabilize centrioles by interacting with their cartwheels and/or with their microtubule walls. In this study we sought to investigate in detail the microtubule binding site of human CEP135/Bld10p (denoted HsCEP135 from here onwards). Based on X-ray crystallography of a Bld10p fragment and of a HsCEP135 fragment, small angle X-ray scattering (SAXS), as well as molecular modeling, we produced an atomic model for the N-terminal domain of HsCEP135. We further defined a microtubule binding site in this domain using biochemical and cryo-EM experiments, and pinpointed by rational mutagenesis three lysine residues that confer microtubule binding of the N-terminal domain of HsCEP135. Our results represent the first high resolution structural information on a CEP135/Bld10p family member and provide insights into how HsCEP135 interacts with microtubules.
RESULTS AND DISCUSSION

**Biophysical and functional characterization of the N-terminal domain of HsCEP135**

Bioinformatic analyses suggested that HsCEP135 is extensively helical and contains several long regions that are predicted to form coiled-coil structures (Figure 2.1A) (Carvalho-Santos et al., 2010; Matsuura et al., 2004). A multiple sequence alignment of CEP135 orthologues revealed a ~160 N-terminal sequence stretch that coincides with a predicted structured region (Carvalho-Santos et al., 2010). In the human and fly proteins, this region of CEP135 has been shown to contain a microtubule-binding site (Carvalho-Santos et al., 2012; Lin et al., 2013). Overall, these observations suggest that the first ~160 N-terminal residues of CEP135 family members form an evolutionarily conserved functional domain that binds microtubules.

To biophysically characterize the N-terminal domain of HsCEP135, we recombinantly expressed and purified the first 158 residues of the protein (denoted HsCEP135-N; Figure 1A). The secondary structure and thermal stability of HsCEP135-N in solution was assessed by circular dichroism (CD) spectroscopy. As shown in Figures 2.1B, the far-UV CD spectrum from HsCEP135-N recorded at 4 °C was typical for an α-helical coiled-coil structure exhibiting minima at 208 and 222 nm, and a [Θ]222:[Θ]208 ratio of >1 (Zhou et al., 1992a, 1992b). Thermal unfolding experiments recorded by CD at 222 nm and at an HsCEP135-N concentration of 10 μM revealed a sigmoidal shaped profile characteristic of a cooperatively folded protein with a midpoint of the transition, Tm, centered at 56 °C (Figure 2.1C; Table 2.1). The oligomerization state of HsCEP135-N was assessed by size exclusion chromatography followed by multi-angle light scattering (SEC-MALS). A molecular mass of 42 kDa was obtained, consistent with the formation of a homodimer (calculated molecular mass of the monomer: 18.7 kDa; Figure 2.1D; Table 2.1).
**Figure 2.1. Characterization of HsCEP135-N.**

(A) Top part, coiled coil (black and gray bars) and secondary structure (white cylinders) prediction of HsCEP135. Black and gray boxes highlight regions with 80-100% and <80% coiled coil probability, respectively. White cylinders depict predicted α-helical regions. Bottom part, schematic representation of protein fragments used in this study. The dashed box highlights the N-terminal domain of HsCEP135. The gray vertical bar highlights segment 96-108, which is crucial for microtubule binding of HsCEP135-N.

(B) and (C) Spectra (B) and thermal unfolding profile (C) recorded by CD from HsCEP135-N (10 μM). The spectra and the unfolding profile were obtained at 4 °C and at 222 nm, respectively. See also Table 2.1. The line in (C) represents the sigmoid fit to the data points.

(D) SEC-MALS experiment obtained by injecting 100 μl of a 200 μM HsCEP135-N protein solution.

(E) Microtubule pelleting assay for HsCEP135-N and representative controls. Shown are relevant areas of Coomassie stained SDS-PAGE gels corresponding to the proteins of interest. S, supernatant; P, pellet; MT, microtubule.
### Table 2.1. Biophysical characterization and microtubule binding activity of CEP135/Bld10p fragments

<table>
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<tr>
<th>Protein</th>
<th>construct</th>
<th>Tm in °C</th>
<th>Calculated MW *a (kDa)</th>
<th>Determined MW *c (kDa)</th>
<th>Oligomerization state</th>
<th>MT binding activity *d</th>
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</thead>
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<tr>
<td>HsCEP135</td>
<td>1-158</td>
<td>56</td>
<td>18.67</td>
<td>43</td>
<td>Dimer</td>
<td>+</td>
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<td>HsCEP135</td>
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<td>-</td>
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</tr>
<tr>
<td>HsCEP135</td>
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<td>7.2</td>
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<td>HsCEP135</td>
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<td>Dimer</td>
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</tr>
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<td>HsCEP135</td>
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<td>+/-</td>
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<td>Dimer</td>
<td>-</td>
</tr>
</tbody>
</table>

*a Determined by far-UV CD  
*b Based on primary amino acid sequence  
*c Determined by SEC-MALS  
*d Determined by microtubule (MT) pelleting assays  
n.d., not determined  
r., experiment performed under reducing buffer conditions  
n.r., experiment performed under non-reducing buffer conditions  

The microtubule-binding activity of HsCEP135-N was tested by a standard microtubule pelleting assay (Campbell and Slep, 2011). As shown in Figure 2.1E, while HsCEP135-N did not pellet on its own, it did so in the presence of microtubules, consistent with previous results (Lin et al., 2013). Inspection by cryo-EM of microtubules incubated with HsCEP135-N revealed that the protein decorated microtubules (Figure 2.2AB), indicating that the interaction between CEP135-N and microtubules is specific. Cryo-tomographic reconstructions of such samples suggested that the decoration most likely reflected the presence of protofilament-based rings and/or spirals that wrap around the microtubule shaft (Figure S2.1AB). Consistent with this hypothesis, analysis of tomograms revealed a
pronounced density with a periodicity of ~8 nm along the long axis of the microtubule, which matches the spacing between tubulin dimers (Figure 2.2CD). We further observed that, besides decorating microtubules, HsCEP135-N can also induce the formation of protofilament-based ring-like structures in the presence of soluble tubulin (Figure S2.1C-E). Interestingly, when tubulin was co-polymerized together with HsCEP135-N, we observed microtubules that were occasionally bridged with either individual straight protofilaments (Figure 2.2E) or with other microtubules (Figure 2.2FG).

Collectively, these results suggest that HsCEP135-N forms a very stable two-stranded coiled-coil structure. They further indicate that in vitro HsCEP135-N binds directly to tubulin, protofilaments and microtubules, and has the capacity to bridge such higher order assemblies. Protofilament-based ring-like oligomers either in isolation or wrapped around microtubules are frequently obtained in the presence of divalent cations or of isolated domains from microtubule-associated proteins (Tan et al., 2006; Wang et al., 2014), and are typically associated with a microtubule depolymerization activity (Desai et al., 1999; Mandelkow et al., 1991); however, we did not find any indication that HsCEP135-N destabilizes microtubules (Figure 2.2AB, see also below). Whether these protofilament-based ring-like oligomers are also formed in the presence of the full length HsCEP135 protein and whether they are of any functional relevance remains to be determined.
Structural model of HsCEP135-N

To provide a structural basis for understanding the interaction of HsCEP135-N with microtubules, we analyzed its structure by X-ray crystallography and in solution by small angle X-ray scattering (SAXS). Crystallization of HsCEP135-N and the corresponding domain from the Chlamydomonas ortholog CrBld10p were not successful. However, after extensive fragment screening, we solved the structures of CrBld10p-N 1-70 and of HsCEP135-N 82-144 to 2.2 and 1.8 Å resolution, respectively (Table 2.2).

As shown in Figure 2.3A, the crystal obtained with CrBld10p-N 1-70 revealed four monomers in the asymmetric unit, which are organized into two individual dimers. The ~20 N-terminal residues of each monomer form short α-helices that assume different orientations presumably as a result of crystal contacts (Figure S2.2A). Residues ~35-60 form a two-stranded parallel and in register coiled-coil structure, where hydrophobic heptad a and d core residues pack in a characteristic ‘knobs-into-holes’ fashion (Lupas and Gruber, 2005; Walshaw and Woolfson, 2001). The N-terminal- and the coiled-coil helices are connected by a ~5-10 amino acid long linker region. The dimeric and cooperatively-folded, helical nature of CrBld10p-N 1-70 was confirmed by CD and SEC-MALS (Figure S2.4AB; Table 2.1).
Table 2.2. Crystallographic table  Data collection, phasing and refinement statistics

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<td>I41 22</td>
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<td>92.74 92.74 164.07</td>
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<tr>
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<td>47.11-2.229 (2.309-2.229)</td>
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<td>17800 (1694)</td>
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<td></td>
<td>72.70</td>
</tr>
<tr>
<td>Water</td>
<td>36.90</td>
<td>56.34</td>
</tr>
<tr>
<td>R.m.s deviations</td>
<td>Bond lengths (Å) 0.002</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Bond angles (°) 0.37</td>
<td>0.56</td>
</tr>
</tbody>
</table>

*Values in parentheses are for highest-resolution shell.
Figure 2.3. Crystal structures of CrBld10p-N 1-70 and HsCEP135-N 82-144.

(A) Crystal structures of the two CrBld10p-N 1-70 dimers found in the asymmetric unit of the crystal and aligned vertically for side to side comparison.

(B) Crystal structures of the single HsCEP135-N 82-144 dimer present in the asymmetric unit of the crystal. The corresponding sequence and structure-based secondary structure assignments are shown below the structures. Residues occupying heptad a and d core positions that pack in a knobs-into-holes fashion are shown in green and orange sticks representation, respectively. Note that the structures in (A) and (B) are shown at a slightly different scale.

See also Figure S2.
To obtain structural information on the N-terminal residues of CrBld10p-N 1-70 in solution, we recorded SAXS data (Figure 2.4A). The calculated pair distribution function (representing a distribution function of inter atomic distances) revealed a distinct peak centered at 20 Å and a maximum particle distance, $D_{\text{max}}$, of 86 Å (Figures 2.4B and S2.3A; Table 2.3), in good agreement with the thickness (22 Å) and elongated nature of a two-stranded coiled-coil structure. Notably, the experimentally determined pair distribution function did not match any of the ones calculated from the homo-dimeric configurations modeled from the four monomer structures present in the asymmetric unit of the crystal (Figures 2.3A and 2.4B), suggesting that the ~30 N-terminal residues of CrBld10p-N 1-70 are indeed largely disordered in solution.

**Figure 2.4.** SAXS analysis of CrBld10p-N 1-70 and HsCEP135-N.

(A) SAXS raw data of CrBld10p-N 1-70 recorded at a protein concentration of 2.5 (red profile) and 5 mg/mL (blue profile). The black profile corresponds to the merged data that were used for further processing.

(B) Distance distribution functions calculated from the experimental SAXS data (black profile) and from the four possible dimeric homoconformeric CrBld10p-N 1-70 atomic models (blue, red, green and orange profiles) that were derived from the crystallographic data (Figure 2.3.A).

(C) SAXS raw data of His-HsCEP135-N recorded at 0.625 (gray profile), 1.25 (red profile) and 2.5 mg/mL (blue profile). The black profile corresponds to the merged data that were used for further processing.

(D) Distance distribution function calculated from the experimental SAXS data of His-HsCEP135-N. Error bars are depicted as gray vertical lines.

See also Figure S3.
This conclusion is further supported by the corresponding ab initio calculated envelope and Kratky plot that displays a significant increase in I*q^2 at higher q values (Figure S2.3BC) characteristic of proteins containing disordered regions (Putnam et al., 2007). HsCEP135-N 82-144 resulted in crystals with two monomers in the asymmetric unit. The monomers formed a two-stranded parallel and in register coiled-coil structure (Figure 2.3B), consistent with the CD analysis of HsCEP135-N 82-144 in solution at 4 °C (Figure S2.2B). Knobs-into-holes packing was observed for heptad a and d core residues spanning segment ~80-130. Notably, Cys110 at a heptad d core position of HsCEP135-N 82-144 forms a disulfide bridge with Cys110’ from the neighboring chain (Figure 2.3B).

Next, we analyzed His-HsCEP135-N by SAXS (Figure 2.4C). As shown in Figure 2.4D, the corresponding calculated distance distribution function revealed a similarly distinct peak centered at 24 Å as obtained for CrBld10p-N 1-70, in agreement with the approximate thickness of a two stranded coiled-coil structure. It further suggested the presence of a highly elongated molecule with a Dmax of 213 Å (Table 2.3). In line with this conclusion, the corresponding Kratky plot and the ab initio calculated envelope indicate a predominantly elongated and folded protein (Figure S2.3DE). However, the course of the Kratky plot in the high q range indicates the presence of some disorder; based on our data obtained CrBld10p-N 1-70, we attributed this disorder to the N-terminal residues of the protein. The molecular masses derived from the SAXS data using the Rambo-Tainer method (Rambo and Tainer, 2013) and from the volume of the molecular SAXS envelope amounts to 40 and 54 kDa, respectively, in good agreement with the calculated molecular mass for the His-HsCEP135-N dimer (40.6 kDa; Table 2.3). In order to generate a full atomic model of HsCEP135-N, we performed homology modeling followed by molecular dynamics simulations using the crystal structures of CrBld10p-N 1-70 (42 and 53% sequence identity and similarity, respectively, to HsCEP135-N 1-70) and HsCEP135-N 82-144 as a basis (Figure 2.5A). Consistent with the SAXS data, the resulting HsCEP135-N model shows an extended homo-dimeric coiled-coil structure ~190 x ~25 Å in size (Figure 2.5B). The theoretical scattering data calculated from the HsCEP135-N model shows a distance distribution function with a similar peak and Dmax as the distance distribution function derived from the His-HsCEP135-N SAXS data and a fit of the theoretical scattering curve to the experimental with a goodness of fit (χ) measure of 1.21 (Figures 2.5C and S2.3F). The fit is particularly good in the low q range that contains the general molecular shape information, and deviates in the high q region that contains more detailed feature information (Putnam et al., 2007).
Figure 2.5. Atomic model of HsCEP135-N.
(A) Root Mean Square Deviation (RMSD) of C$_\alpha$ atoms of HsCEP135-N. Only residues 82-133 were used for the calculation as the N- and C-terminus of HsCEP135-N are flexible.
(B) Simulated structure of HsCEP135-N with the Pro70 residue highlighted (magenta). Pro70 introduces a kink in the structure consistent with the ab initio SAXS envelope. A representative conformation from the largest cluster is shown.
(C) Distance distribution function calculated from the experimental SAXS data of His-HsCEP135-N (black profile) and from the atomic model of HsCEP135-N (blue profile) that was derived by modeling and MD-simulation.
(D) Overlay of the atomic model of HsCEP135-N from the modeling with the best His-HsCEP135-N DAMMIN envelope (NSD 0.509) (Figure S2.3.E).
(E) Surface view of the HsCEP135-N model color-coded with the electrostatic surface potential calculated at pH 6.8 (from -10 to +10 k$_B$T; red and blue depict negative and positive electrostatic potentials, respectively). Regions of positive electrostatic potential are indicated and numbered with 1, 2 and 3.
See also Figure S2.3
Notably, the HsCEP135-N model shows a bend that is caused by a proline residue in the sequence (Pro70). A bend at the corresponding position is also observed in the *ab initio* envelope generated from the SAXS data (Figure 2.5BD), and is supported by the fact that prolines can introduce kinks in coiled-coil structures (Chang et al., 1999). The overall conformation of the model fits well into the SAXS envelope and matches the observed SAXS scattering data obtained from His-HsCEP135-N (Figures 2.5CD and S2.3F). Further analysis of the HsCEP135-N atomic model revealed three patches along the coiled coil (denoted 1, 2 and 3), which display strikingly high positive electrostatic surface potentials (Figure 2.5E).

Collectively, our biophysical and structural data suggest that the predominantly positively charged N-terminal domain of HsCEP135 forms an extended two-stranded coiled-coil structure with chains arranged in parallel and in register. They further indicate that the first ~25-30 N-terminal residues of HsCEP135 are largely disordered in solution and adopt an ensemble of different conformations.

**Table 2.3.** SAXS data processing using in house software

<table>
<thead>
<tr>
<th>Protein</th>
<th>construct</th>
<th>Concentration (mg/mL)</th>
<th>(R_G (\text{Å}))</th>
<th>(D_{\text{max}} (\text{Å}))</th>
<th>Calculated MW(^2) (kDa)</th>
<th>Determined MW (kDa) by SAXS</th>
<th>Oligomerization state</th>
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<tr>
<td>HsCEP135</td>
<td>His-tagged 1-158</td>
<td>2.5</td>
<td>60.2</td>
<td>170-202</td>
<td>20.03</td>
<td>37.4(^1)</td>
<td>Dimer</td>
</tr>
<tr>
<td>HsCEP135</td>
<td>His-tagged 1-158</td>
<td>1.25</td>
<td>60.4</td>
<td>180-208</td>
<td>20.03</td>
<td>41.4(^1)</td>
<td>Dimer</td>
</tr>
<tr>
<td>HsCEP135</td>
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<td>60.4</td>
<td>180-210</td>
<td>20.03</td>
<td>42.3(^1)</td>
<td>Dimer</td>
</tr>
<tr>
<td>HsCEP135</td>
<td>His-tagged 1-158</td>
<td>average</td>
<td></td>
<td></td>
<td>20.03</td>
<td>24.1(^2)</td>
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<td>CrBld10p</td>
<td>1-70</td>
<td>5</td>
<td>24.7</td>
<td>70-80</td>
<td>7.95</td>
<td>16.9(^1)</td>
<td>Dimer</td>
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<tr>
<td>CrBld10p</td>
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<td>24.1</td>
<td>75-85</td>
<td>7.95</td>
<td>17.1(^1)</td>
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<td></td>
<td></td>
<td>7.95</td>
<td>23.0(^2)</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Determined using the Rambo and Tainer method (Rambo and Tainer, 2013)
\(^2\) Determined using BSA as a standard

\(R_G\): Radius of Gyration
**Identification and characterization of a microtubule binding site in HsCEP135-N**

We set out to narrow down the microtubule-binding region in HsCEP135-N. For this purpose, we generated several N- or C-terminal truncations and recombinantly expressed and purified the resulting fragments (Figure 2.1A). The structural integrity of the various fragments was assessed by CD and SEC-MALS. As shown in Figure S2.4 and summarized in Table 2.1, like the full length HsCEP135-N domain, most of the fragments thereof formed dimeric coiled-coil structures. An exception was HsCEP135-N 96-158, which was monomeric in reducing buffer conditions; however, under non-reducing conditions, HsCEP135-N 96-158 formed a stable coiled-coil dimer. This result can be explained by the presence of a cysteine residue at a heptad d core position in HsCEP135-N (Cys110), which allows for the formation of a disulfide bridge in the context of a two-stranded parallel and in register coiled-coil structure (Chernyatina and Strelkov, 2012). This analysis shows that all HsCEP135-N fragments produced preserved the two-stranded coiled-coil structure of the full length protein under appropriate buffer conditions.

Next, the microtubule-binding activities of the dimeric HsCEP135-N fragments were tested using microtubule pelleting assays. As summarized in Table 2.1 and shown in Figure 2.6A, the two minimal HsCEP135-N fragments 1-108 and 96-158 retained the ability to pellet together with microtubules. This observation suggests that a microtubule-binding site is located between residues 96-108. Notably, this segment coincides with the positively charged surface patch 2 identified in our atomic HsCEP135-N model (Figure 2.5E). It is well known that clusters of basic amino acids of microtubule-binding domains are frequently implicated in mediating interactions with the negatively charged outer surface of microtubules (Ciferri et al., 2008; Fourniol et al., 2010; Maurer et al., 2012). We thus tested the functional relevance of selected basic residues in this region. Inspection of the HsCEP135-N 82-144 crystal structure revealed three lysine residues, K101, K104 and K108, which are present in the microtubule-binding region 96-108 (Figure 2.6BC) and which are responsible for the high positive electrostatic surface potential of patch 2 (Figures 2.5E and 2.6C). To test the relevance of these three lysine residues we simultaneously mutated them to alanine in HsCEP135-N 96-158 (HsCEP135-N 96-158 3xK), HsCEP135-N 68-158 (HsCEP135 68-158 3xK), HsCEP135-N 1-108 (HsCEP135 1-108 3xK) and HsCEP135-N (HsCEP135-N 3xK). Analysis by CD and SEC-MALS demonstrated that all four triple mutants maintained dimeric coiled-coil structures in solution (Figure S2.4 and Table 2.1; HsCEP135-N 96-158 3xK was assessed under non-reducing buffer conditions).
The microtubule-binding activities of the various HsCEP135-N mutants were assessed by microtubule pelleting assays. In contrast to wild type, HsCEP135-N 96-158 3xK did not pellet together with microtubules (Figure 2.6A), suggesting that residues K101, K104 and K108 are important for microtubule binding of this HsCEP135-N fragment. HsCEP135-N 68-158 3xK and HsCEP135-N 1-108 3xK retained some microtubule binding activity as indicated by the weaker bands in the corresponding pellet fractions (Figure 2.6A). Intriguingly, HsCEP135-N 3xK still pelleted together with microtubules, suggesting that this mutant retained the capacity to somehow interact with microtubules as tested in this assay (Figure 2.6A).

![Figure 2.6. Identification of a microtubule-binding site in HsCEP135-N.](image)

(A) Microtubule pelleting assays for the indicated HsCEP135-N fragments and mutants. Shown are relevant areas of Coomassie stained SDS-PAGE gels. S, supernatant; P, pellet.

(B) Close-up view of the microtubule-binding site in the crystal structure of HsCEP135-N 82-144 (cartoon representation). Residues K101, K104 and K108 that were simultaneously mutated are shown in sticks representation. The 2Fo-Fc electron density map (blue mesh) contoured at 1σ is superimposed onto the structure.

(C) Surface view of HsCEP135-N 82-144 color-coded with the electrostatic surface potential calculated at pH 6.8 (from -10 to +10 kT; red and blue depict negative and positive electrostatic potentials, respectively). See also Figure S2.4.
To investigate these observations further, we conducted immunofluorescence experiments of microtubules that were mixed with either HsCEP135-N 3xK or HsCEP135 96-158 3xK (i.e., two mutants showing either binding or no binding in pelleting assays) or with their respective wild type variants, before being spun onto coverslips and stained with antibodies against α-tubulin and HsCEP135-N. As shown in Figure 2.7A, microtubules did not significantly pellet on their own in this assay; however, in the presence of wild type HsCEP135-N, crosslinked and bundled microtubules were present on the coverslip (Figure 2.7B). This effect is reminiscent of classical microtubule-stabilizing proteins (Kanai et al., 1992; Lewis et al., 1989) or of proteins that stabilize microtubules by inducing bundling upon overexpression (Bu and Su, 2003; Hoogenraad et al., 2000), suggesting that HsCEP135-N has a stabilizing effect on microtubules in vitro. By contrast, microtubules, while present, were much less crosslinked/bundled with one another in the presence of HsCEP135-N 3xK (Figure 2.7C). Similar experiments carried out with the HsCEP135-N 96-158 variant established that whereas the wild-type fragment interacted with microtubules, no microtubules were spun onto the coverslip in the presence of HsCEP135-N 96-158 3xK (Figure 2.7D; note that the HsCEP135-N antibodies do not recognize the HsCEP135-N 96-158 fragment). These results are fully consistent with the ones obtained with the microtubule pelleting assay (Figure 2.6A) and concur to establish that residues K101, K104 and K108 are critical for efficient microtubule crosslinking/bundling by HsCEP135-N.

Collectively, these results narrow down a major microtubule-binding site in HsCEP135-N to a 13 amino acid region (segment 96-108). They further identify three surface exposed lysine residues in this segment, which confer the microtubule-crosslinking/bundling activity of the HsCEP135-N in vitro; however, they also indicate that additional elements flanking the segment 96-108 contribute to the overall microtubule-binding affinity of HsCEP135-N. Based on our current mutagenesis data, we suspect that such elements are likely to be present on the N-terminal side of the segment 96-108, since the mutant HsCEP135-N 96-158 3xK failed to interact with microtubules in pelleting assays. Thus, it is likely that the dimeric structure in combination with multiple microtubule-binding sites per monomer is the source of the microtubule-crosslinking/bundling activity of HsCEP135-N.
Figure 2.7. Immunofluorescence microscopy of microtubules in the absence and presence of HsCEP135-N variants.

(A-D) Fluorescence microscopy of microtubules pelleted alone or in the presence of indicated wild-type or mutant HsCEP135-N variants, and revealed by immunofluorescence microscopy with α-tubulin and HsCEP135 antibodies. Scale bars, 5 μm. Note that the HsCEP135-N antibodies do not recognize epitopes in the 96-158 region, and are thus not shown in (D).
Conclusions

There is a general agreement that CEP135/Bld10p promotes the assembly and stability of the centriolar microtubule wall and hence of the entire centriole organelle from algae to human. However, in the absence of structural information prior to this work, how this protein family exerts this role at a mechanistic level has remained elusive. Here, by combining structural, biophysical and biochemical approaches we have characterized in detail the structure of the N-terminal domain of human CEP135/Bld10p in vitro. We found that HsCEP135-N forms a very stable, parallel two-stranded coiled-coil structure. Our results further suggest that the ~30 N-terminal residues of HsCEP135-N are possibly disordered in solution. Interestingly, these residues belong to a ~40 amino acid stretch that is the most conserved region among the members of the CEP135/Bld10p family of proteins (Carvalho-Santos et al., 2010). The present data do not reveal the function of this most N-terminal segment; due to its high conservation across evolution, we expect that this part of CEP135/Bld10p may represent a binding site for centriolar protein partner(s) that awaits identification.

It has been recently reported that there is a short human CEP135/Bld10p splice isoform (dubbed CEP135mini) comprising the first 249 N-terminal residues of HsCEP135, plus 16 amino acids that differ in sequence from the full length protein (Dahl et al., 2015). This shorter HsCEP135 variant negatively regulates centriole assembly by inhibiting the activity of the full-length protein, presumably by preventing it from interacting with centriolar binding partners such as HsSAS-6 (Dahl et al., 2015). Notably, our HsCEP135-N atomic model accounts for the first approximately two-thirds of CEP135mini. It is well established that coiled-coil proteins can readily exchange their chains (Lehrer et al., 1989). One possibility to explain the inhibitory effect of CEP135mini is thus that it forms heterodimers with HsCEP135 and in this way negatively regulates the activity of the full-length protein.

Our study demonstrates that the N-terminal part of HsCEP135 binds tubulin, straight and curved protofilaments, and microtubules in vitro; to the best of our knowledge, this is the first demonstration that a coiled-coil domain can bind different types of tubulin assemblies. Our results further reveal that HsCEP135-N can crosslink/bundle and thus stabilize microtubules, which can be explained by the homodimERIC structure of HsCEP135-N harboring multiple microtubule-binding sites. The capacity of HsCEP135-N to simultaneously bind two or more microtubules could contribute to the formation of microtubule triplets or else to linking neighboring triplets within the centriolar microtubule.
wall. In this context, it has been reported that HsCEP135 binds CPAP (Lin et al., 2013), an essential centriolar protein that interacts with tubulin and the $\gamma$-tubulin complex and which is crucially involved in centriole biogenesis (Hung et al., 2000; Kohlmaier et al., 2009; Schmidt et al., 2009; Tang et al., 2009). A next goal will be to define how full length HsCEP135 is spatially organized in the lumen of centrioles, and how and to which extent its N-terminal domain collaborates with additional protein partners to control the assembly and stability of the centriolar microtubule wall.
EXPERIMENTAL PROCEDURES

Sequence analysis
Secondary structure predictions were performed using PSIPRED (Buchan et al., 2013). For the prediction of unstructured regions, DisEMBL was used (Linding et al., 2003). Coiled-coil prediction was performed using MARCOIL (Delorenzi and Speed, 2002). Sequence searches, analyses and alignments were performed using BLAST (Camacho et al., 2009) and ClustalW 2.1 (Larkin et al., 2007).

Cloning and protein preparation
Cloning of HsCEP135 (UniProtKB-Q66GS9) and CrBld10p (UniProtKB-Q764P7) constructs was performed by the restriction free positive selection method using the PSTCm1 vector (Olieric et al., 2010). For introducing PCR amplified inserts into the linearized vector co-transformation into the E. coli strain Mach1 (Invitrogen) was performed. Mutations were introduced using the QuickChange mutagenesis approach (Stratagen).

All recombinant proteins contained an N-terminal 6xHis-tag for affinity purification followed by a thrombin cleavage site. For standard expression, the proteins were transformed into the E.coli expression strains BL21(DE3) or BL21(DE3)RP (Stratagen). Transformed cells were incubated at 37 °C until an OD_{600} of 0.4 to 0.6. The cultures were subsequently cooled down to 20 °C prior to induction with 0.5 mM iso-propyl thiogalactiside (IPTG). Expression was carried out overnight at 20°C. Cells were harvested by centrifugation at 4 °C for 15-20 min. Cell pellets were washed once with 20 mM Tris-HCl, pH 7.5, supplemented with 150 mM NaCl and 2 mM DTT, and stored at -20 °C until further use.

All protein purification and processing was performed at 4 °C. Cells were lysed either by sonication or using an EmulsiFlex-C3 homogenizer (Avestin) in HEPES, pH 8.0, supplemented with 500 mM NaCl, 10 mM imidazole, 2 mM beta-mercaptoethanol, 10 mM MgSO_4 and an EDTA-free protease inhibitor cocktail (Roche). His-tagged proteins were purified on HiTrap Ni^{2+}-Sepharose chelating columns (Amersham) following the manufacturer’s instructions. 6xHis-tags were cleaved in thrombin cleavage buffer (20 mM Tris-HCl, pH 8.4, supplemented with 150 mM NaCl, 2.5 mM CaCl_2) for 18 h using 10 units of thrombin per 5 mg/mL protein. A second affinity chromatography step was performed to
remove the uncleaved protein, the 6xHis-tag and further impurities. A final purification step was performed on either S200 16/60 or S75 16/60 (depending on the size of the proteins) size exclusion chromatography columns (GE Healthcare) in 20 mM Tris-HCl, pH 7.5, supplemented with 150 mM NaCl, 1 mM DTT. Protein samples were concentrated to ~10 mg/mL, flash frozen and stored at -80 until further use.

**CD spectroscopy**
For CD spectroscopy, protein samples were diluted to a final concentration of 10 or 20 µM depending on the expected signal strength in PBS buffer at pH 7.5 containing 300 mM NaCl. For measurements under reducing conditions, 2 mM TCEP or 2 mM DTT were added to the protein samples. Far-ultraviolet (UV) CD spectra recorded at 4 °C and thermal unfolding profiles recorded at 222 nm were obtained on a Chirascan spectrophotometer (AppliedPhotophysics) equipped with a temperature control unit and sample temperature sensors. The samples were measured in a quartz cell of 0.1 cm or 0.05 cm path length. A step ramping rate of 1 °C and an equilibration time of 30 sec was used to record the thermal unfolding profiles. The apparent midpoints of the transition, T_m’s, were determined by fitting of the data points using the R nonlinear least square fitting function based on a sigmoid model.

**SEC-MALS**
SEC-MALS experiments were performed in 20 mM Tris-HCl, pH 7.5, supplemented with 150 mM NaCl, 1 mM DTT using an S-200 10/30 analytical SEC column connected in-line to miniDAWN TREOS light scattering and Optilab T-rEX refractive index detectors (Wyatt Technology). Measurements were carried out at 20 °C and at a flow rate of 0.4 mL/min. Data analysis was performed using the software package provided by the vendor. Concentrations of the proteins were as follows: HsCEP135 1-158, 0.4 mg/mL; 1-158 3xK, 0.4 mg/mL; 1-108, 2 mg/mL; 1-97, 4 mg/mL; 68-158, 2 mg/mL; 96-158, 9 mg/mL; 96-158 3xK, 4.5 mg/mL; 1-70, 1.2 mg/mL; 96-158, 5 mg/mL; CrBld10p 1-70, 2.5 mg/mL. Injection volume was 100 µL for each experiment.

*Microtubule pelleting assay and immunofluorescence analysis*
Standard microtubule pelleting assays were performed (Campbell and Slep, 2011). Briefly, Taxol-stabilized microtubules (8 µM tubulin mixed with 4 µL of 20, 200 and 2000 µM Taxol
successively added to 800 μL final sample volume) polymerized in BRB80 buffer (80 mM PIPES-KOH, pH 6.8, 1 mM MgCl₂, 1 mM EGTA) were sedimented by centrifugation at 195’000 g and at 37 °C for 20 min and resuspended in reducing agent free BRB80 buffer. Subsequently, the microtubules were incubated at 25 °C for 20 min in a final volume of 100 μL with 20 μM of a given HsCEP135-N protein sample. The mixture was subsequently centrifuged at 195’000 g and at 25 °C for 20 min through a 150 μL glycerol cushion (BRB80 supplemented with 0.02 mM Taxol, 40% glycerol). Pellets and supernatants were analyzed by Coomassie stained 15% SDS-PAGE gels. As controls, either tubulin, microtubules or HsCEP135-N proteins alone were processed the same way.

For immunofluorescence analysis, 10 μM Taxol-stabilized microtubules were mixed with an equimolar amount of HsCEP135-N variants and spun down on a coverslip for 10 min at 10’000 g. Samples were then fixed in ice-cold methanol for 5 min followed by wash in PBS and incubation with blocking solution (1% w/v BSA in PBS, 0.5% Tween-20, PBT) for 30 min. Primary and secondary antibodies were diluted in PBT as follows: mouse anti-α-tubulin (clone DM1A, Sigma-Aldrich, St. Louis, MO): 1/1000; rabbit anti-HsCEP135 1-158 (Meritxell Orpinell and Pierre Gönczy, unpublished): 1/500; goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 568 (both Life Technologies, Grand Island, NY): both 1/1000. Primary antibodies were incubated at 4 °C overnight and secondary antibodies at room temperature for 1 h, and the slides were washed with PBT. All images were processed with ImageJ (Schneider et al., 2012).

**Electron microscopy**

Specimens were prepared by incubating Taxol-stabilized microtubules (20 μL of 10 μM tubulin in BRB80, prepared similarly as for the microtubule pelleting assay) with an equimolar amount of HsCEP135-N variants. Then, 2 μL of a 100 μM solution of soluble tubulin was added to the mix after 5 min. of incubation to prevent formation of microtubule bundles. Tubulin ring-like oligomers were formed by mixing 10 μL of 100 μM soluble tubulin with equimolar amounts of HsCEP135-N 68-158. Bridged microtubules were obtained by mixing and incubation of an equimolar amount of free tubulin with HsCEP135 68-158 for 30 min. followed by addition of 0.2 mM Taxol final concentration and further incubation for 1 h at 37°C. Protein samples were adsorbed onto Lacey carbon film grids (300 microMesh), blotted with a filter paper (Whatman) and vitrified in liquid ethane using a homemade plunging apparatus.

Samples were imaged with a FEG Tecnai F 20 (FEI Eindhoven, The Netherlands)
transmission electron microscope operated at 200 kV and equipped with a cryo-specimen grid holder Gatan 626 (Warrendale, PA). Images were acquired at 29,000 x using an Eagle camera (4096 x 4096 pixels, binning 2, 0.748 nm final pixel size). For cryo-tomography, the same setting were used to acquire tilt series covering an angular range from -60° to +60° with 2° degrees increments and 4–5 microns defocus. The tomograms were aligned using 10 nm fiducial gold markers (BSA Tracer 10 nm, AURION) and reconstructed by R-weighted back projection using the IMOD software package (Kremer et al., 1996). The resulting stacks were analyzed using ImageJ (Schneider et al., 2012) and displayed in 3D using UCSF chimera (Pettersen et al., 2004).

**Crystallization and X-ray data collection**

CrBld10p 1-70 (54 mg/mL) crystals were obtained at 20 °C in 0.1 M phosphate citrate, pH 4.0, 5% 2-propanol and 0.2 M lithium sulfate. Cryoprotection was achieved by addition of 20% glycerol. HsCEP135 82-144 (15 mg/mL) crystals were obtained in a commercial PACT screen (Qiagen) 25% PEG 1500, SPG buffer, pH 6.0 at 20 °C. All data sets were collected at 100 K at the X06DA and X06SA beamlines at the Swiss Light Source (Paul Scherrer Institute). For the CrBld10p 1-70 sulfur SAD data at different Chi angle offsets (0°, 10°, 20°, 30°, 40°) were collected at 2.0664 Å wavelength.

**Structure solution**

All data sets were reduced, scaled and merged using XDS, XSCALE and XDSCONV (Kabsch, 2010a, 2010b). For CrBld10p 1-70, phases were obtained by sulfur SAD phasing using SHELX via the HKL2MAP interface (Pape and Schneider, 2004; Schneider and Sheldrick, 2002). Automatic model building was done with BUCCANEER (Cowtan, 2012). Refinement was performed by alternating rounds of model building using COOT (Emsley et al., 2010) and refinement with Phenix.refine from the PHENIX suite (Adams et al., 2010).

For HsCEP135-N 82-144, the phase problem was solved by molecular replacement using the coiled-coil structure of GCN4 (PDB ID 4GKW) as a search model. After obtaining an initial PHASER (McCoy et al., 2007) solution the model was first improved manually based on the electron density using the model building program COOT (Emsley et al., 2010), then chain traced with SHELXE (Thorn and Sheldrick, 2013) and built with BUCCANEER (Cowtan, 2012). Refinement was performed as for CrBld10p 1-70. See Table 2.2. for all data collection and refinement statistics.
Electrostatic surface potentials were calculated using APBS (Baker et al., 2001). The binning scale in the units of $k_BT$ was chosen for the range from -10 to +10. The protonation state at pH 6.8 (the pH used to perform the microtubule pelleting assays) was determined using PDB2PQR (Dolinsky et al., 2007). The analysis of knobs into holes packing of coiled-coil structures was carried out using SOCKET (Walshaw and Woolfson, 2001). Secondary structure elements were assigned using STRIDE (Frishman and Argos, 1995).

**SAXS measurements**

SAXS measurements were performed at the cSAXS (X12SA) beamline at the Swiss Light Source (Paul Scherrer Institute) and at the BM29 bio-SAXS beamline at the European Synchrotron Radiation Facility (Pernot et al., 2013) at 10 and 20 °C, respectively. All measurements were taken at 1 Å wavelength. The q value is defined as $4\pi \sin \theta / \lambda$, where $\theta$ is the scattering angle.

Prior to the measurements, all samples were either spun down or filtered to remove possible aggregates. Concentrations ranging from 0.3 to 10 mg/mL for His-HsCEP135-N and from 1.25 to 5 mg/mL for CrBld10p 1-70 were measured. Data were analyzed in parallel using the ATSAS software suite (Petoukhov et al., 2012) and using an in house software package. Merging and cutting of the data was performed with PRIMUS (Konarev et al., 2003). The distance distribution functions were calculated using GNOM (Svergun, 1992) and were used as input for the DAMMIF/DAMMIN ab initio shape reconstruction (Franke and Svergun, 2009). Calculated distance distribution functions were generated with GNOM from simulated scattering data that were computed from atomic models with CRYSOL (Svergun et al., 1995).

**Molecular modeling and Molecular dynamics simulation**

An atomic model of the HsCEP135-N was built using Modeller 9v14 (Webb and Sali, 2014) using our crystal structures of CrBld10p-N 1-70 and HsCEP135-N 82-144. Missing residues 71-81 and 145-158 were modeled in an α-helical configuration by introducing restraints on symmetry and secondary structure, as the protein forms a homo-dimeric coiled-coil structure. A set of 50 models were generated and evaluated using the Discrete Optimized Protein Energy (DOPE) values. The model with the best DOPE value was then further energy minimized and subjected to molecular dynamics simulations.

The model was first solvated in water and the charge of the system was then
neutralized by adding 12 Cl⁻ ions. The final system comprised 450’890 atoms (1 HsCEP135-N, 148’246 waters, and 418 K⁺ and 430 Cl⁻ ions). The complete system was equilibrated at 310 K and 1 atm pressure for 24 ns. The model was simulated for an additional 72 ns. All the simulations were performed using the CHARMM force field (Mackerell, 2004) in the NAMD2.10 package (Phillips et al., 2005). Trajectories were visualized using VMD (Humphrey et al., 1996) and clustering based on RMSD was performed. A representative conformation from the largest cluster was used for figure preparation and comparison to the SAXS data. The theoretical scattering curve of the generated model was computed and compared with the program CRYSOL (Svergun et al., 1995) using the default parameters. The model was aligned with the SAXS envelope of HsCEP135-N 1-158 via SUPCOMB (Kozin and Svergun, 2001).
Supplementary Figure 2.1, related to Figure 2.2. Cryo EM analysis of HsCEP135-N bound to microtubules.

(A) and (B) Cryo-tomogram section through a microtubule decorated with HsCEP135-N. Note that HsCEP135-N induces the formation of tubulin ring-like oligomers that wrap around the microtubule (red arrows). Two such structures are visible and form a continuous line characteristic of closed protofilament-based ring-like oligomers. These structures can also be seen in the 3D representations (red arrows in panel B).

(C) Cryo-EM micrograph of tubulin ring-like oligomers obtained in the presence of HsCEP135-N 68-158 and free tubulin.

(D) Cryo-tomogram section showing top (green arrow) and side views (red arrow) of tubulin ring-like oligomers. Note that rings are composed of several layers.

(E) Gallery showing top views of tubulin ring-like oligomers.
Supplementary Figure 2.2 related to Figure 2.3. Analysis of the crystal structures of CrBld10p 1-70 and CD spectra of HsCEP135-N 82-144.

(A) Cartoon representation of the CrBld10p 1-70 structure in the asymmetric unit (blue) of the crystal and its surrounding symmetry mates (grey).

(B) Far-UV CD spectra recorded at 4 °C of HsCEP135-N 82-144 (10 μM).
Supplementary Figure 2.3, related to Figure 2.4 and 2.5. SAXS data.
(A) Distance distribution function of CrBld10p 1-70. Error bars are depicted as gray lines.
(B) Kratky plot of CrBld10p 1-70.
(C) Best DAMMIN envelope by NSD (normalized spatial difference: obtained after a 10x repeated refinement of 20 initial DAMMIF runs) for CrBld10p 1-70; NSD 0.659.
(D) Kratky plot of HsCEP135-N.
(E) Best DAMMIN envelope by NSD (normalized spatial difference: obtained after a 10x repeated refinement of 10 initial DAMMIF runs) for His-HsCEP135-N; NSD 0.509.
(F) Calculated SAXS scattering curve of the atomic model of HsCEP135-N from the modeling (black line) fitted to the experimentally derived and merged SAXS scattering curved of His-HsCEP135-N (red line; Figure 4C). Chi-value: 1.21.
Supplementary Figure 2.4, related to Figure 2.6. Biophysical characterization of HsCEP135-N protein variants used in this study.

(A) Far-UV CD spectra recorded at 4 °C of the following HsCEP135-N variants: orange, HsCEP135-N 1-108 (10 μM); blue, HsCEP135-N 1-97 (10 μM); red, HsCEP135-N 1-70 (10 μM); green, HsCEP135-N 68-158 (10 μM); magenta, HsCEP135-N 96-158 (20 μM under reducing buffer conditions); grey, crBld10p 1-70 (10 μM).

(B) Thermal unfolding profiles recorded by CD spectroscopy at 222 nm and sigmoidal fit for the following HsCEP135-N variants: orange, HsCEP135-N 1-108 (10 μM); blue, HsCEP135-N 1-97 (10 μM); red, HsCEP135-N 1-70 (10 μM); green HsCEP135-N 68-158 (10 μM); grey, CrBld10p 1-70 (10 μM).

(C) Far-UV CD spectra recorded at 4 °C and at 10 μM protein concentration of HsCEP135-N 3xK (black), HsCEP135 1-108 3xK (green), HsCEP135 68-158 3xK (blue), and HsCEP135 96-158 3xK (red).

(D) Thermal unfolding profiles recorded by CD spectroscopy at 222 nm (10 μM protein concentration) and sigmoidal fit for HsCEP135-N 3xK (black), HsCEP135 1-108 3xK (green), HsCEP135 68-158 3xK (blue), and HsCEP135 96-158 3xK (red).

(E) Thermal unfolding profiles recorded by CD spectroscopy at 222 nm and sigmoidal fit for HsCEP135 96-158 (20 μM) under reducing (black circles) and non-reducing conditions (black triangles).
Chapter 3
SAS-6 engineering reveals interdependency between cartwheel and microtubule wall in determining centriole architecture
INTRODUCTORY NOTE
The data presented and discussed in this chapter are the authors contribution (if not stated otherwise) to the project resulting in the following publication:


SAS-6 engineering reveals interdependence between cartwheel and microtubules in determining centriole architecture

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2Department of Biological Sciences, University of Tokyo, Tokyo, Japan
3Swiss Institute for Experimental Cancer Research (ISREC), School of Life Sciences, Swiss Federal Institute of Technology (EPFL), Lausanne, Switzerland
4Department of Biosystems Science and Engineering, Eidgenössische Technische Hochschule (ETH) Zürich, Basel, Switzerland
5Condensed Matter Theory Group, Paul Scherrer Institut, Villigen PSI, Switzerland.
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7Current address: Department of Frontier Bioscience, Hosei University, Koganei, Tokyo, Japan
8These authors contributed equally to the work

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INTRODUCTION

SAS-6 is a crucial component of the centriole architecture and as related in Chapter 1.2 is suggested to generate a scaffold which determines the centriole symmetry. This scaffold hypothesis predicts that a change in cartwheel symmetry would result in a change of centriole symmetry. The biophysical and bioinformatical analysis by Dr. M Hilbert and Dr. M. Pfreudschuh on the Chlamydomonas SAS-6 showed that crSAS-6 foremost adopts 9-fold symmetric arrangements \textit{in vitro} as measured by atomic force microscopy (AFM) (Hilbert et al., 2016; Kitagawa et al., 2011c). Besides the dominating 9-fold symmetry (43 %), also 8-fold (30%) and 10-fold (27 %) symmetric arrangements were found. Molecular dynamics simulations by Dr. M Hilbert using the crSAS-6 crystal structure (Kitagawa et al., 2011c) supported the notion that the variability in symmetric arrangements is rooted in an inherent flexibility of the crSAS-6 N-terminal domain dimerization interface. This led to the assumption that the cartwheel symmetry could be manipulated by introducing mutations in this dimerization interface (Figure 3.1.A).

As shown in Figure 3.1.B the N-terminal dimerization interface is characterized by an hydrophobic interaction. F145 is fitting in a pocket generated by I95 and F102 of the neighboring SAS-6 N-terminal domain. This hydrophobic interaction was probed by substituting F145 with various hydrophobic residues and was named hydrophobic contact approach.

A further approach was designed to strengthen the native salt-bridge interactions by K105 and D144 and to enlarge the salt-bridge network (Figure 3.1.C). To increase the rigidity of the interface even further, a third approach was developed focusing on the introduction of covalent bonds by disulfide bridge formation (named the covalent linkage approach). During the first round of design, mutants exclusively based on one single strategy were generated (Hilbert et al., 2016). Subsequently, after the biophysical characterization of those mutants, the hydrophobic contact and the salt-bridge network strategy were combined during a second round of mutation as well as several salt-bridge network mutations (NN23). For a full list of the mutations see Table 3.1.
Figure 3.1. CrSAS-6 N-terminal domain dimerization interface. Figure adapted from Hilbert et al., 2016.

(A) Overview of CrSAS-6 N-terminal domain forming the biological dimer.

(B) to (D) Close-up views showing the NN dimer interface. The residues considered for mutation are depicted in stick representation. B) 'hydrophobic contact', C) 'salt bridge' and D) 'covalent linkage' mutagenesis approaches. All mutants are listed in Table 3.1.
<table>
<thead>
<tr>
<th>Name</th>
<th>Mutations</th>
</tr>
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<td>WT</td>
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</table>

**Hydrophobic contact**
NN1  | F145M               |
NN2  | F145W               |
NN3  | I95F                |
NN4  | I95V F102W          |
NN5  | F102W F145Y         |
NN21 | I95V                |

**Salt bridge**
NN6  | K105R               |
NN7  | K105R D144E         |
NN8  | N143R               |
NN10 | Q93E                |
NN12 | Q93E K146R          |
NN13 | G94D Q147K          |
NN14 | G94E Q147K          |
NN15 | G94D Q147R          |
NN16 | G94E Q147R          |
NN17 | H150R               |

**Covalent linkage**
NN18 | K105C F145C         |
NN19 | G94C K146C          |
NN20 | I95C K146C          |

**Combination of hydrophobic contact and salt bridge or several saltbridge mutations**
NN23 | Q93E G94D K146R     |
|      | Q147R              |
NN24 | Q93E F145W K146R    |
NN25 | G94D F145W Q147R    |
NN26 | G94E F145W Q147K    |
NN27 | Q93E F145W          |
|      | Q93E G94D F145W     |
|      | K146R Q147K         |

Table adapted from Hilbert et al., 2016
n.d., not determined.

All mutants were named “NN” for N-terminal domain followed by a number reflecting the order in which they were designed. In cases were the nomenclature refers to a specific biological dimer the chain identifiers are given as part of the mutant name. Characterization of the mutants included the measurement of the Kd values of the N-terminal dimerization interface (Hilbert et al., 2016) and determination of the in vitro symmetry via AFM and EM Figure 3.2. (The data for the Figure was assembled an generated by Dr. M. Hilbert, the Figure was edited by the author.)
RESULTS AND DISCUSSION

In order to rationally explain the observed effects crystallization and structure determination for all mutants was attempted by Dr. M. Hilbert and the author. The proteins were expressed, purified and crystallized as described in method section of this chapter and in Hilbert et al. (Hilbert et al., 2016). The data was collected at the Swiss Light Source (Paul Scherrer Institute; Switzerland) beamlines X06DA and X06DS. All structures were phased via molecular replacement using the previously published native N-terminal domain structure of CrSAS-6 (Kitagawa et al., 2011 PDB ID: 3Q0Y). In total 9 mutant structures were determined, 4 from the first round of mutations (NN2, NN10, NN18, NN19) and 5 from the second round of mutations (NN23, NN24, NN25, NN26, NN27) Table 3.2. While for NN2 the hydrophobic contact strategy was employed, NN10 and NN23 are based on the optimization of the salt-bridge network. The mutants NN24-NN27 were based on a combination of hydrophobic contact and salt-bridge network improvement. The number of monomers per asymmetric unit, biological dimers, and RMSDs are listed in Table 3.3. In case, were several biological dimers were found per asymmetric unit, their divergence was determined by aligning the Cα traces of the biological dimers (Table 3.3.). While for NN19 and the wildtype structure (PDB ID: 3Q0Y) the RMSD among the biological dimers identified in the structures is below 1 Å, it is between 1.6 and 1.9 Å for the remaining mutants. In general, the RMSD to the reference wildtype biological dimer is in between 0.7 Å an 2.2 Å, whereas the alignment of NN24AA with the reference structure resulted in an RMSD of 4 Å (Table 3.3.).
### Table 3.2. X-ray data collection and refinement statistics

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*Values in parentheses are for highest-resolution shell.
Table 3.3. Asymmetric unit content of mutant CrSAS-6 structures

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<th>Number of monomers per asymmetric unit</th>
<th>Number of biological dimers per asymmetric unit</th>
<th>RMSD between the first biological dimer and the additional ones in the asymmetric unit in Å.</th>
<th>RMSD between the wildtype dimer formed by chain C and D and the indicated dimers in Å.</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt (CD, AB, EF)</td>
<td>4</td>
<td>2</td>
<td>0.7, 0.5</td>
<td>0.0, 0.7, 0.5</td>
</tr>
<tr>
<td>NN2 (AD, BC)</td>
<td>2</td>
<td>1</td>
<td>1.6</td>
<td>1.3, 2.1</td>
</tr>
<tr>
<td>NN18 (AB)</td>
<td>1</td>
<td>created by symmetry mate</td>
<td>n.a.</td>
<td>0.7</td>
</tr>
<tr>
<td>NN19(AB, CD, EF)</td>
<td>6</td>
<td>3</td>
<td>0.8, 0.5</td>
<td>0.8, 0.4, 0.7</td>
</tr>
<tr>
<td>NN23(AB, CD)</td>
<td>4</td>
<td>2</td>
<td>1.8</td>
<td>1.2, 2.2</td>
</tr>
<tr>
<td>NN24 (AA)</td>
<td>1</td>
<td>created by symmetry mate</td>
<td>n.a.</td>
<td>4.0</td>
</tr>
<tr>
<td>NN25(AB)</td>
<td>2</td>
<td>1</td>
<td>n.a.</td>
<td>2.4</td>
</tr>
<tr>
<td>NN26(AB, CD)</td>
<td>4</td>
<td>2</td>
<td>1.9</td>
<td>1.5, 2.0</td>
</tr>
<tr>
<td>NN27(AB, CD)</td>
<td>4</td>
<td>2</td>
<td>1.8</td>
<td>1.30, 2.2</td>
</tr>
</tbody>
</table>

n.a.: not available

a: Aligned were the Cα atoms of the biological dimers.

Note that data was also collected for NN28 and NN12. Phasing and preliminary refinement was possible. While the data and the resulting models were good enough to derive the overall orientation of the domains, they were not sufficient to be published in the PDB and thus were not further considered for analysis.

In general, the structures show that the salt-bridge mutations resulted in a slightly elevated number of polar contacts for NN26, and NN27 and an increased number of polar contacts in the dimeric interface for NN23, NN24, and NN25 compared to the wildtype structure (Kitagawa et al., 2011); PDB ID: 3Q0Y,) (Table 3.4). For all analyzed polar contacts the average contact length ranged from 2.8 to 3.0 Å. The number of polar contacts was reduced for the cystein mutants NN18 (average: 8) and NN19 (average: 5.3).

Table 3.4.: Number and average length of polar contacts

<table>
<thead>
<tr>
<th>Name</th>
<th>Number of contacts in the interface (per biological dimer in the crystal structure)</th>
<th>Average contact length in Å (per biological dimer in the crystal structure)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>10, 10, 10</td>
<td>2.9, 2.9, 2.9</td>
</tr>
<tr>
<td>NN2</td>
<td>11, 10</td>
<td>3.0, 2.9</td>
</tr>
<tr>
<td>NN10</td>
<td>12</td>
<td>3.0</td>
</tr>
<tr>
<td>NN18b</td>
<td>8</td>
<td>3.0</td>
</tr>
<tr>
<td>NN19b</td>
<td>5, 5, 6</td>
<td>2.8, 2.9, 2.9</td>
</tr>
<tr>
<td>NN23</td>
<td>13, 16</td>
<td>3.0, 2.9</td>
</tr>
<tr>
<td>NN24</td>
<td>12</td>
<td>2.9</td>
</tr>
<tr>
<td>NN25</td>
<td>12</td>
<td>3.0</td>
</tr>
<tr>
<td>NN26</td>
<td>11, 10</td>
<td>2.9, 3.0</td>
</tr>
<tr>
<td>NN27</td>
<td>10, 11</td>
<td>2.9, 2.8</td>
</tr>
</tbody>
</table>

a: Mutant with improved hydrophobic contacts
b: Cystein mutants to facilitate covalent linkage
In the close up views (Figure 3.3.) the impact of the mutations is visualized for representative dimeric interfaces of the corresponding crystal structures. As shown in Figure 3.3.A, in NN2 (F145W) the introduced W145 oriented towards the hydrophobic pocket of the neighboring N-terminal domain. For NN10 the introduced mutations resulted in observable salt-bridge interactions as expected (Figure 3.3.B). Salt bridges, hydrogen-bonds and polar contacts were assigned with the build in PYMOL function “cmd.dist()” using a distance cutoff of 3.6 Å.

In case of NN18 and NN19 cystein point mutations were introduced in order to facilitate the formation of disulfide bridges (Figure 3.3.CD). Only in NN18 the cysteins are in the right spacial arrangement and distance of approximately 2.05 Å (3.3 Å for the alternative conformation of C105, respectively) to form disulfide bridges (Petersen et al., 1999). In all NN19 biological dimers derived from the crystal structure the interatomic distance of the sulfur atoms from C94 to C' 146 and vice versa ranges between 7.2 to 7.8 Å and thus is too large for disulfide bridge formation (Petersen et al., 1999). As the B-factors of C94 and C146 (ranging from 48 to 53 Å² depending on the chain) are close to the average B-factors of NN19 (49 Å², see Table 3.2.) it is unlikely that they possess a high flexibility enabling a closure of the gap without larger rearrangements.

Figure 3.3.E-I show representative interfaces of the mutants resulting from the combinations of the “hydrophobic contact” and the “salt-bridge network” strategy as well as NN23 which combines several mutations to improve its salt-bridge network. For all those mutants polar interactions generated by the introduced mutations could be identified.

Apart from the biological dimers identified in the structures of NN18, NN23, NN24, NN25 and NN26, all biological dimers have a total buried surface area upon dimer formation between 1500 Å² and 1600 Å² similar to the values calculated for the wildtype dimers (Table 3.5.). NN18 has the smallest total buried surface area (1249 Å²) of all analyzed dimers. NN23, NN25 and NN26 on the other hand display total buried surface area values ranging from 1720 Å² to 1840 Å². NN24 stands out with a total buried surface area of 2081 Å². The enlarged total buried surface area for the second round mutants indicates that the combination of strategies and mutations indeed resulted in increased interactions and larger interfaces for most of them (NN27 excluded) (Table 3.5.).
Figure 3.3. Crystal structures of NN dimer mutants.

Close-up views of representative dimer interfaces of mutant CrSAS-6 NN dimer variants solved by X-ray crystallography (see also Table 3.2.). Mutated residues are shown in stick representation and are colored in orange (monomer 1) and blue (monomer 2). Residues in the vicinity of the mutated residues are shown in black (monomer 1) or gray (monomer 2). Black dotted lines indicate polar contacts as identified with PyMol.
Table 3.5: Total buried surface area upon formation of the biological dimers.

<table>
<thead>
<tr>
<th>Biological dimer</th>
<th>Buried surface area in Å². a</th>
</tr>
</thead>
<tbody>
<tr>
<td>wtAB</td>
<td>1520</td>
</tr>
<tr>
<td>wtCD</td>
<td>1530</td>
</tr>
<tr>
<td>wtEF</td>
<td>1520</td>
</tr>
<tr>
<td>NN2AD</td>
<td>1560</td>
</tr>
<tr>
<td>NN2BC</td>
<td>1590</td>
</tr>
<tr>
<td>NN10AB</td>
<td>1550</td>
</tr>
<tr>
<td>NN18AA</td>
<td>1249</td>
</tr>
<tr>
<td>NN19AB</td>
<td>1580</td>
</tr>
<tr>
<td>NN19CD</td>
<td>1530</td>
</tr>
<tr>
<td>NN19EF</td>
<td>1560</td>
</tr>
<tr>
<td>NN23AB</td>
<td>1720</td>
</tr>
<tr>
<td>NN23CD</td>
<td>1830</td>
</tr>
<tr>
<td>NN24AA</td>
<td>2081</td>
</tr>
<tr>
<td>NN25AB</td>
<td>1730</td>
</tr>
<tr>
<td>NN26AB</td>
<td>1790</td>
</tr>
<tr>
<td>NN26CD</td>
<td>1840</td>
</tr>
<tr>
<td>NN27AB</td>
<td>1550</td>
</tr>
<tr>
<td>NN27CD</td>
<td>1590</td>
</tr>
</tbody>
</table>

a: calculated with the PISA or taken from annotated PDB file (Krissinel and Henrick, 2007)

The projected symmetry change was evaluated by successively aligning the solved NN biological dimer structures (and the NN dimer structures of the wildtype (wt) for reference) with the wildtype NN dimerization-impaired coiled-coil dimer structure (PDB ID: 3Q0X) (Figure 3.4., Figure 3.5.). The symmetry of the assemblies was determined as 360° divided by the angle between the two vectors connecting the center of masses of their respective NN dimers.

In general, the determined symmetries differ only to a small extent from the distributions found by AFM (Figure 3.2.). Nevertheless, the biological dimers forming flat rings NN2BC, NN23CD, NN26CD and NN27CD have a symmetry lower than their observed range in vitro (Figure 3.2. and 3.5.). Interestingly, NN24 has a lower projected symmetry for the constructed ring-like complex than the other combined strategy mutants NN23, NN25, NN26, and NN27 that were structurally assessed (Figure 3.5.). Since NN24 and NN25 share the same number of polar contacts (12, 12) at a comparable average contact length (2.9, 3.0) (Table 3.4.), it is likely that the lower NN24 symmetry is caused by improved hydrophobic contacts in the dimer interface, as it displays the highest total buried surface area of all analyzed constructs (Table 3.5).
Furthermore, as for the wildtype (Figure 3.4.), the reconstruction of the ring-like complex for structures containing several biological dimers per asymmetric unit (NN2, NN19, NN23, NN26, NN27) showed that the same mutant can sample different helical pitches and ring-symmetries. This indicates an intrinsic flexibility in the dimeric interface.

Figure 3.4. Ring-complex reconstructions for the crBld10 wildtype.
A)-C) Reconstruction of the expected ring complexes based on the biological dimers identified in the CrBld10 wildtype structure (PDB code:3Q0y). The chain identifiers are given after the construct name(wt). The calculate symmetry is denoted with “Sym:”. 
To evaluate this intrinsic flexibility an anisotropic network model analysis (ANM) was performed for all NN mutant biological dimers as well as the wildtype (PDB ID: 3Q0Y). After the Hessian matrix was build for the full corresponding protein C-alpha track, the model was reduced to the set of residues common to all structures (prior to recalculation of the modes) to allow a comparison of the modes of different biological dimers. The models were reduced according to Hinsen et al. (Hinsen et al., 2000).

To compare the dynamicity of the biological dimers the root mean square inner product (RMSIP) and the covariance overlap were calculated (Amadei et al., 1999; reviewed in Fuglebakk et al., 2015; Hess, 2002). While the former provides a measure for the agreement of the directionality concerning the harmonic movement, the later provides a measure for the magnitude and the directionality (Table 3.6). Considered for comparison were only the first twenty, non trivial modes. All calculated ANMs were compared to the ANM calculated for the wildtype biological dimer formed by the chains C and D (named wtCD), as its projected ring-like complex symmetry is closest to the mean projected symmetry of 10.03 for the wild type structure (Figure 3.4.).

Table 3.6.: Anisotropic Network Model analysis for the biological dimers

<table>
<thead>
<tr>
<th>Biological dimer</th>
<th>Root mean square inner product to wtDC biological dimer as reference.</th>
<th>Covariance overlap to wtDC biological dimer as reference.</th>
</tr>
</thead>
<tbody>
<tr>
<td>wtAB</td>
<td>0.90</td>
<td>0.66</td>
</tr>
<tr>
<td>wtEF</td>
<td>0.98</td>
<td>0.84</td>
</tr>
<tr>
<td>NN2AD</td>
<td>0.96</td>
<td>0.80</td>
</tr>
<tr>
<td>NN2BC</td>
<td>0.84</td>
<td>0.52</td>
</tr>
<tr>
<td>NN10AB</td>
<td>0.93</td>
<td>0.71</td>
</tr>
<tr>
<td>NN18A</td>
<td>0.93</td>
<td>0.75</td>
</tr>
<tr>
<td>NN19AB</td>
<td>0.89</td>
<td>0.60</td>
</tr>
<tr>
<td>NN19CD</td>
<td>0.98</td>
<td>0.88</td>
</tr>
<tr>
<td>NN19EF</td>
<td>0.97</td>
<td>0.80</td>
</tr>
<tr>
<td>NN23AB</td>
<td>0.96</td>
<td>0.78</td>
</tr>
<tr>
<td>NN23CD</td>
<td>0.83</td>
<td>0.50</td>
</tr>
<tr>
<td>NN24AA</td>
<td>0.74</td>
<td>0.34</td>
</tr>
<tr>
<td>NN25AB</td>
<td>0.95</td>
<td>0.72</td>
</tr>
<tr>
<td>NN26AB</td>
<td>0.97</td>
<td>0.80</td>
</tr>
<tr>
<td>NN26CD</td>
<td>0.73</td>
<td>0.27</td>
</tr>
<tr>
<td>NN27AB</td>
<td>0.97</td>
<td>0.82</td>
</tr>
<tr>
<td>NN27CD</td>
<td>0.87</td>
<td>0.55</td>
</tr>
</tbody>
</table>
For most mutant biological dimers the 20 lowest frequency modes are comparable to the ones observed for the reference wildtype dimer in terms of directionality and magnitude (RMSIP values ~0.9; covariance overlap > 0.6). However, while for NN2BC, NN23CD, NN24AA, NN26CD and NN27CD the RMSIP values range from 0.73-0.87, the covariance overlap values ranging from 0.27-0.55 indicate a difference in the calculated modes compared to the reference. While for NN24 such a difference in dynamicity (RMSIP: 0.74; covariance overlap: 0.34) could possibly explain the low ~5-fold projected symmetry (Figure 3.5.J), NN2BC, NN23CD, NN26CD and NN27CD all have projected symmetries of at least 6-fold (Figure 3.5.). However, NN2BC, NN23CD, NN26CD and NN27CD do not form helical oligomers as the other analyzed dimers, but rather flat rings (Figure 3.5.). This difference in the dynamic behavior for NN mutants forming flat rings of ca. 6-fold symmetry (symmetry observed in vitro was mostly 8-fold Figure 3.2.) could be caused by additional conformational changes due to crystal contacts. As discussed above these biological dimers differ all by more than 2 Å in RMSD from the wildtype reference and have a slightly larger total buried surface area than their counterparts generating helical oligomers (Table 3.3. and 3.5.). In fact, the other biological dimers appearing in the corresponding asymmetric units are closer in RMSD to the reference structure than they are to these flat-ring conformers (Table 3.4.). This could indicate that the extreme conformations of these chains and their different dynamic behavior are crystal artifacts. Nevertheless, due to the additional biological dimers in the asymmetric units of the respective crystal structures we know that these mutants are capable of sampling other conformations and projected symmetries.

NN24AA on the other hand is forming helical arrangements as expected based on the wildtype structures(Figure 3.4. and 3.5.J). NN24AA (biological dimer generated via symmetry mate) shows the second largest divergence in RMSIP and covariance overlap from the modes calculated for the reference dimer. It differs by an RMSD of 4 Å from the reference structure (Table 3.3.) and comprises the largest dimerization interface (Table 3.5.). As NN24AA shows consistent divergence in all analyzed measure from the wildtype reference, the observed projected and measured in vitro symmetry of mostly 5-6-fold (Figure 3.2 and 3.5J) is likely to be caused by the difference in conformation and dynamic behavior rooted in the introduced mutations.

However, since no additional biological dimers were solved it is not clear whether the full conformational space is described by the ANM of NN24. Nevertheless, generally ANMs
successfully sample sufficient conformational space to describe slow harmonic molecular motions (Krebs et al., 2002; reviewed in Lopez-Blanco et al., 2007).

**CONCLUSION**

The structure based evaluation of the generated mutants has shown that we successfully altered the symmetry of the SAS-6 oligomeric structure by rational mutation of the interface residues. Using experimentally determined structures and computational evaluation of the protein structure dynamicity we could confirm the effect of the rational introduced mutations. Interestingly, the ANM calculations indicated differences in the dynamic behavior for the biological dimers NN2BC, NN23CD, NN24AA, NN26CD and NN27CD derived from the respective crystal structures. For NN24AA the difference seems to be genuinely caused by the mutated interface residues as it still forms helical arrangements, but differs strongly in projected symmetry and RMSD from the wildtype reference structure and is consistent with the symmetry measured in vitro (Figure 3.2.). However, the flat ca. 6-fold symmetric rings of NN2BC, NN23CD, NN26CD and NN27CD could be caused either by additional conformational changes due to crystal contacts or could represent extreme conformations. Although normally the starting structures are treated as being in equilibrium in ANM modeling and therefore no initial energy minimization is needed (reviewed in Hayward and Groot, 2008), it would be interesting to see whether energy minimization or molecular dynamics simulation of the biological dimers could lead to a conformation similar to those observed for the helix forming dimers observed in the respective crystal structures.

The mutants NN2, NN24 and NN26 (together with NN15) were introduced and evaluated in *C. reinhardtii* by the group of Dr. H. Masafumi (Department of Frontier Bioscience, Hosei University, Koganei, Tokyo, Japan). A human SAS-6 mutant comparable to NN24 was introduced in human cells by the group of Prof. P. Gönczy(Swiss Institute for Experimental Cancer Research (ISREC), School of Life Sciences, Swiss Federal Institute of Technology (EPFL), Lausanne, Switzerland) (Hilbert et al., 2016). The aim was to determine whether the alterations of the oligomer symmetry observed in vitro and in the structure analysis were transferable to the full centriole in cells.

Interestingly, only the NN24 mutant could be established in *C. reinhardtii* and was therefore the only mutant considered for experiments in human cells. Notably, most of the
identified *C. reinhardtii* centrioles did not contain cartwheels in context of the NN24 mutant. Those that did displayed foremost 9-fold symmetry and a few displayed 8-fold symmetry (Hilbert et al., 2016). As this is different from the centriole symmetry observed for the CrSAS-6 knock out strain *bld12* null (for which 10-11-fold symmetry could be observed), we concluded that CrSAS-6 indeed has an impact on the centriole symmetry. However, besides the fact that no symmetry lower than 8-fold was observed (as it was in vitro), severing the link between the cartwheel and the microtubule wall by truncating the protein Bld10p resulted in different symmetries for the cartwheel and the microtubule wall. Interestingly, no impact on centriole symmetry was observed for NN24 equivalent mutants expressed in human cells, rather a reduction in centriole length. This led us to the conclusion that two distinct, but interdependent mechanisms, one based on the cartwheel and one based on the microtubule wall, are determining the centriole symmetry. For a full description of additionally performed experiments see (Hilbert et al., 2016).
METHODS

Note: Only methods that were directly applied to generate the data presented in this chapter are described in this section. For the description of further methods important to the overall study see Hilbert et al., (Hilbert et al., 2016).

Part of the herein presented analysis was re-performed by Dr M. Hilbert using a different set of software for the final paper draft. Thus the exact values can vary from those stated here.

Crystallization

Crystallization was performed in 96 well plates as sitting drop vapor diffusion experiment using conditions similar as for the wildtype (Kitagawa et al., 2011c). Fine screening was performed using 200 nL protein solution and 200 mother liquor. Screening was carried out using a Phoenix robot. All crystallization experiments were carried out at 293 K. The exact conditions are provided in Table 3.7.

Table 3.7.: Crystallization conditions

<table>
<thead>
<tr>
<th>Name of construct</th>
<th>Crystallization conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>NN2</td>
<td>0.1 M MES pH6, 20% PEG6000</td>
</tr>
<tr>
<td>NN10</td>
<td>0.1 M MES pH6.5, 24% PEG3350</td>
</tr>
<tr>
<td>NN18</td>
<td>0.1 M MES pH6, 16% PEG3350</td>
</tr>
<tr>
<td>NN19</td>
<td>0.1 M MES pH6 20% PEG6000</td>
</tr>
<tr>
<td>NN23</td>
<td>0.1 M MES MONONHYDRATE PH 6.0, PEG 3350</td>
</tr>
<tr>
<td>NN24</td>
<td>0.1 M MES pH6.5 22% PEG3350</td>
</tr>
<tr>
<td>NN25</td>
<td>0.1 M MES pH6.5 14% PEG3350</td>
</tr>
<tr>
<td>NN26</td>
<td>0.1 M MES pH6 12% PEG3350</td>
</tr>
<tr>
<td>NN27</td>
<td>0.1 M MES pH6.5 14% PEG3350</td>
</tr>
</tbody>
</table>

Data acquisition and processing

Diffraction data was collected at the Swiss Light Source macromolecule beamlines X60DA and X60SA at 100 K. Prior to data collection crystals were stepwise soaked in mother liquor supplemented with up to 20% glycerol for cryoprotection.

All data was reduced and processed using the programs XDS, XSCALE and XDSCONV (Kabsch, 2010a, 2010b).
Phasing and refinement
All structures were phased by molecular replacement using the program PHASER (McCoy et al., 2007) as provided in the PHENIX suite (Adams et al., 2002) with the native N-terminal dimer structure as a search model (PDB code: 3Q0Y). Subsequent rounds of manual model building and refinement were carried out with COOT (Emsley et al., 2010) and Phenix.refine from the PHENIX suite (Adams et al., 2002), respectively.

Structure analysis
The structures were analyzed using the program PyMol (The PyMOL Molecular Graphics System, Version 1.7.2.1 Schrödinger, LLC.) and its build in functions for the determination of polar contacts, alignments and structure analysis. This was performed utilizing PyMols python API, which allowed an automation of the analysis using PYTHON (V. 2.7) scripts. The dimerization interface was defined as all residues in a 3 Å radius around the residues considered for mutation (see Figure 3.1). Polar contacts were identified using the “cmd.dist()” command of PyMol, which considers the geometry and distance to assign polar contacts. The chosen distant cutoff value was 3.6 Å.

The ring-like complexes were generated by repeatedly aligning copies of the solved NN mutant structures with the N-terminal domain of copies from the N-terminal dimerization impaired CrSAS-6 structure (PDB ID: 3Q0X).

The angle and symmetry was estimated from this ring like oligomers using the angle between two Vectors, each connecting the center of masses of a NN-dimer. The position of the center of mass was determined using the PyMol build in function “centerofmass()”. As the exact value calculated for the angle and therefore the projected symmetry depends on the exact location of the center of mass, which in turn depends of the function in use for its determination, the projected symmetry values can only be considered estimates.

All structure images were prepared using PyMol (The PyMOL Molecular Graphics System, Version 1.7.2.1 Schrödinger, LLC.). The average B-factors for the residues were calculated using the BioPython module (Cock et al., 2009) in a PyMol session invoked from a PYTHON script.

The total buried surface area upon dimer formation was retrieved from the REMARK 350 sections of the corresponding PDB files (automatically generated with PISA upon deposition) or calculated with the webserver PISA ('Protein interfaces, surfaces and assemblies' service PISA at the European Bioinformatics Institute.) (Krissinel and Henrick, 2007).
Anisotropic network model calculation and analysis

ANM modes were calculated in a PYTHON (V. 3.4) script using the ProDy python module designed for protein structure and dynamic analysis (Bakan et al., 2011). The ANM model was reduced using the “reduceModel()” function in ProDy, which was implemented according to Hinsen et al. (Hinsen et al., 2000). The implemented functions in ProDy for the calculation of the root mean square inner product and the covariance overlap have been derived according to Amandi et al. and Hess, respectively (Amadei et al., 1999; Hess, 2002). The application of these measures for comparing the intrinsic dynamic has been discussed by Fuglebakk et al. (Fuglebakk et al., 2015).
Chapter 4
General discussion and outlook
The centrosome is an important cell organelle and is fundamental for cellular functions such as cell division and organization of the cytoskeleton network. At its core reside two centrioles in perpendicular arrangement surrounded by pericentriolar material. The centrosome and the centrioles follow their own duplication cycle, during which the build up of their distinct architecture is achieved. The centrioles consist of a hollow cylinder generated by a 9-fold symmetric assembly of microtubule multiplets. In the proximal lumen a cartwheel-like assembly connects to the centriolar microtubule wall. The 9-fold symmetric cartwheel consists of a central hub with nine spokes radiating outwards. The spokes are connected to the centriolar microtubules by the so called pinhead region at the end of the spokes. Several studies revealed a set of key proteins that is involved in the build up and/or maintenance of the centriolar architecture. One essential key protein is SAS-6/Bld12p, which is the main component of the cartwheel. Structural studies showed that SAS-6/Bld12p can self-assemble in vitro into 9-fold symmetric ring-like oligomers with outwards oriented coiled-coil spokes. These oligomers are comparable in their appearance to the centriolar cartwheel. Nevertheless, how the spokes of SAS-6/Bld12p are connected to the centriolar microtubules and to which degree SAS-6/Bld12p imposes the 9-fold symmetry remained elusive. To answer these questions, we investigated the potential linker protein CEP135/Bld10p. Furthermore, we applied a rational mutagenesis approach to SAS-6/Bld12p to achieve an alteration of the cartwheel symmetry.

The microtubule binding ability of CEP135/Bld10p is a prerequisite for its attributed functions of linking the cartwheel directly to the centriolar microtubules (for certain organisms) and of stabilizing them. To characterize the microtubule binding of the human CEP135 N-terminus we combined a wide range of structural methods with biochemical assays. We further investigated to which degree SAS-6/Bld12p influences the centriolar 9-fold symmetry. For that purpose the dimeric interface of the SAS-6/Bld12p N-terminal domain was modified by rational mutations to alternate binding strength and oligomer symmetry. The resulting mutants were evaluated by X-ray crystallography and biophysical means. Studies in human cells and C. reinhardtii were performed with the most promising mutants.

Our studies revealed that human CEP135 consists of a kinked, parallel dimeric coiled-coil connected by a loop to a flexible α-helix at the N-terminus of each monomer. The coiled-coil section of the CEP135/Bld10p N-terminus harbors the microtubule binding site, which contains three lysines important for microtubule binding. We further succeeded in identifying several mutants which alter the symmetry of the SAS-6/Bld12p ring oligomers.
Interestingly, introduction of selected mutants into cells led only to minor alterations of the centriolar symmetry. Only when the connection between cartwheel spokes and the centriolar microtubules was severed in *C. reinhardtii* by depleting Bld10p, the mutations altered the symmetry of the cartwheel. This suggests the existence of at least two interdependent mechanisms for the generation of the centriolar 9-fold symmetry, one based on the cartwheel and one based on the microtubule wall.

In conclusion, new questions can be posed in context of our findings. The possibility of further microtubule binding sites at the CEP135 N-terminus needs to be addressed as well as the location of the CEP135-binding site on microtubules. Furthermore, how CEP135 can be arranged in the restricted space of the proximal centriolar lumen remains an open question. In general, the impact of our findings on the proposed functions of CEP135/Bld10p and SAS-6/Bld12p has to be evaluated.

### 4.1. Additional microtubule binding sites in the N-terminus of HsCEP135

As concluded in Chapter 2, the identified microtubule binding site of HsCEP135 contains three lysines (K101, K104, K108), which generate a surface area of strong positive electrostatic potential. Combined mutations of the lysines to alanines resulted in different effects for different constructs. While the mutant construct HsCEP135 96-158 3xK did not bind to microtubules, the mutant HsCEP135 1-158 3xK still bound to microtubules, albeit microtubule bundling was reduced. Although these results demonstrate the importance of the mutated lysine residues, they also raise the question of potential further microtubule or tubulin binding sites within the N-terminal domain of CEP135. Thus, retained microtubule interaction ability of the HsCEP135 1-158 3xK could be due to different reasons: Unspecific aggregation of tubulin and microtubules might be caused by charged patches due to construct artifacts.

The microtubule binding region is larger than initially identified and thereby retains part of its binding ability in the HsCEP135 1-158 3xK construct.

1) The electrostatic long range interaction mediated by the lysines are unspecifically attracting the negative charged microtubule surface, while for actual binding further residues in the interface may be important.

2) An additional binding site might be located in the construct HsCEP135 1-158.

Concerning point 1) it is noteworthy that the first 70 amino acids of HsCEP135 also display a strong positive electrostatic surface potential, although this construct did not bind
microtubules. Therefore, a strong positive electrostatic potential might be beneficial, albeit insufficient to cause unspecific microtubule binding in this context. Nevertheless, positively charged residues and strong positive electrostatic potential as well as cations have been described as the cause for unspecific microtubule interaction in the literature (Diaz et al., 1996; Wang et al., 2014).

It is remarkable that the construct 96-158 3xK does not show a tendency to co-sediment with microtubules. Therefore, neither a strong unspecific interaction nor a more extensive binding region is likely to be present in the range from 96-158. In addition, the constructs 1-70 and 1-97 do not bind to microtubules, which excludes an extension of the binding site in direction of the N-terminus. The contribution of additional important residues in the binding site (point 3), seems equally unlikely, due to the lack of microtubule binding for HsCEP135 96-158 3xK.

An additional binding site might be located in the construct HsCEP135 1-158. However, if one excludes the regions 1-70 and 1-97 as possible regions for a binding site (due to the results of the pelleting assays), the only region left to be explored resides C-terminal of the identified binding site, i.e., amino acid range 109-158. Under the premises that an additional binding site would display similar characteristics as the identified one in terms of amino acid composition and spacing of the positively charged residues, the residues 129 to 136 can be postulated as potential second binding site. They follow an inverted pattern “K/R-X-X-X-K/R-X-X-X-K/R” compared to the “K/R-X-X-K/R-X-X-X-K/R” pattern at the identified binding site. The potential of that second binding site can be tested with a construct that only comprises this site. This may require extending the C-terminus of the construct beyond amino acid 158 in order to obtain a stable construct.

Another possibility is to generate a mutant construct without the potential second binding site, e.g., 1-127, to examine residual co-sedimentation. However, it remains that HsCEP135 96-158 3xK did not co-sediment with microtubules. Therefore, a second binding site located in the amino acid range from 108-158 is unlikely.

Since the smaller constructs seem to exclude the effects observed with the HsCEP135 1-158 3xK concerning additional binding activity, it could be argued that HsCEP135 1-158 forms a back-folded entity which is disrupted in the different truncation constructs. However, the SAXS data show that HsCEP135 is of elongated conformation without any indication for a more complicated fold vulnerable to truncation. Whether CEP135 contains microtubule or tubulin binding sites outside of the N-terminal domain (1-158) remains to be tested, but the truncation data from Lin et al. suggest otherwise (Lin et
Microtubule binding activity of CEP135 was shown for *D. melanogaster* as well as for *H. sapiens* in cells (Carvalho-Santos et al., 2012; Lin et al., 2013) and the centriolar position of the *C. reinhardtii* Bld10p N-terminus in the pinhead region suggests likewise for this ortholog (see Chapter 1.2.). It seems likely that the microtubule binding observed for HsCEP135 is a general feature of this protein family. However, due to low sequence identity for CEP135 between different clades, microtubule binding cannot easily be inferred based on sequence alignments. Furthermore, CEP135 seems to be absent in *C. elegans* (Carvalho-Santos et al., 2010). Therefore, the evaluation of further orthologs is necessary to determine the spread and the potential consensus sequence of the CEP135 microtubule binding site.

### 4.2. Further structural investigations of CEP135 microtubule binding.

Although the microtubule binding site was identified on CEP135, the site on the microtubules where CEP135 binds remains enigmatic. CEP135 may bind to 1) the lateral or longitudinal interdimer interfaces in between tubulin dimers or 2) to the tubulin dimers directly. Furthermore, whether the microtubule binding sites on each monomer in a CEP135 coiled-coil are independent or interdependent remains to be elucidated. This could be investigated with short monomeric peptides of the CEP135 microtubule binding site or the monomeric construct HsCEP135 96-158 (monomeric under reducing conditions). The peptide length will have to be chosen carefully to prevent dimerization and to ensure exclusive presence of the monomer in solution. Furthermore, it is possible that either the context of a coiled-coil dimer or at least of an α-helical arrangement is necessary to promote microtubule binding. In case of the former the peptide based approach is out of question, while for the latter the peptide might retain a minimum of α-helical folding or could be designed in a way to mimic the helical distances between the important lysine residues in a linear peptide. Interestingly, the cryo-electron microscopy images reveal that microtubules are bundled by CEP135 (Figure 2.2.) thereby indicating that the two binding sites of a CEP135 dimer can bind to distinct microtubules.

To investigate the position of CEP135 on microtubules an immunofluorescence microscopy based competition assay using *in vitro* polymerized microtubules could be applied. Thereby, microtubules would be decorated with CEP135 stained via fluorescent antibodies. Different characterized microtubule binders, such as the kinesin motor domains.
would be applied to replace CEP135 from the microtubules to assess whether they have coinciding binding sites. Another possible method would be the use of different crosslinking agents with subsequent mass spectrometry analysis to identify tubulin residues involved in binding CEP135 (Petrotchenko and Borchers). This approach would benefit from the use of recombinantly produced tubulin, since tubulin purified from natural sources is heterogeneous (Minoura et al., 2013), which would complicate the subsequent mass spectrometry analysis.

Mutation of the lysines 101, 104 and 108 revealed that these positively charged residues contribute to microtubule binding. This highlights the possibility that CEP135 binds to the flexible C-terminal tails of either α- or β-tubulin or to both tails. To test this, pre-polymerized microtubules can be treated with subtilisin following the method used by Saoudi et al., which results in tail removal prior to the pelleting assay (Saoudi et al., 1995).

Computational docking of CEP135 to a high resolution microtubule EM structure such as the one recently published by Zhang et al. provides a way to combine information from different experimental sources (Zhang et al., 2015). As global docking may lack accuracy and is limited by the size of the system, experimentally guided local docking is to be preferred. Suitable programs for experimentally guided docking would be HADDOCK (Dominguez et al., 2003; van Zundert and Bonvin, 2014), were active and passive residues contributing to the binding site are defined, or RosettaDock (Lyskov and Gray, 2008) were the user prepositions the docking partners according to prior knowledge. Docking in general needs to be rigorously validated to confirm the quality of obtained hits. The docking should be consistent among several runs with differing starting conditions such as different subsets of experimentally identified residues as active residues in case of HADDOCK and should yield experimentally falsifiable hypotheses.

All the methods discussed so far are limited to an indirect deduction of the binding site, which needs to be subsequently confirmed, e.g., by mutagenic studies using recombinant tubulin. To study the binding of CEP135 to microtubules directly a helical or single particle cryo-electron microscopy reconstruction is necessary. In order to accomplish successful averaging homogeneous decoration is required. Similar strategies have been applied for kinesin (Alushin et al., 2014). Combined efforts towards this goal have been made in collaboration with Prof. P. Guichard (University of Geneva), but are still ongoing.
4.3. Possible structure of CEP135/Bld10p in the proximal lumen of the centriole

We demonstrated that the N-terminus of human CEP135 mainly consists of an extended coiled-coil domain with a proline at position 70 that introduces a kink in the otherwise linear tertiary structure. If the full length CEP135 is forming an extended coiled-coil, similar to the N-terminal region, than an approximate length of 167 nm (~20 nm length of 1-158 and 982*0.1495 nm rise per amino acid in a theoretical poly-alanine coiled-coil (Lupas and Gruber, 2005)) would be expected. Centrioles have an inner diameter of about 130 nm (Gönczy, 2012). In Figure 4.1. the expected CEP135 length is set to scale with an EM density map of a *Trichonympha* basal body cartwheel and a microtubule triplet (Figure 4.1.). Assuming the binding of multiple dimers of CEP135 to one centriolar microtubule, even a purely longitudinal (parallel to the protofilaments) arrangement may result in steric clashes. Therefore, a more complicated fold than an extended coiled-coil needs to be considered. By creating a synthesis of the novel insights in the structure of the CEP135 N-terminus with low resolution structural information from the current literature, a set of restraints can be derived for possible arrangements of CEP135 in the proximal centriolar lumen. It was shown in Chapter 2 that CEP135 is predicted to consist of long regions of coiled-coil tertiary structure (mostly coinciding with predicted α-helical regions) connected by short unstructured regions. Indeed, the structural model for the CEP135 N-terminus derived in Chapter 2.2. confirms this notion at least for this region. The range of amino acids 1-158 consists of a short flexible α-helix at the far N-terminus connected by a hinge region to a parallel coiled-coil domain. The region around Pro70 induces a kink in the coiled-coil domain. Extrapolating from the structure of the CEP135 N-terminus and based on the secondary structure prediction (Chapter 2.2), CEP135 can be described as an array of rigid, parallel, dimeric coiled-coil domains connected by more or less structured regions of various length. These regions would allow for kinks, hinges, bends or even for backfolding and stacking.
Since the determined microtubule binding site resides within a region of parallel dimeric coiled-coil, this stretch of coiled-coil has to be located in close contact to the centriolar microtubules. For microtubules decorated with HsCEP135-N (1-158) a ~ 8 nm periodicity of decoration was observed. As the 1-158 region spans approximately 20 nm along its long axis, the question arises which arrangement of CEP135 allows for an 8 nm periodicity without causing steric clashes. Thus, all purely horizontal or vertical arrangements may be excluded in context of a full decoration of all protofilaments in vitro (Figure 4.2.).
Kleylein-Sohn et al. could clearly show via immunogold labeling electron microscopy that the C-terminus of human CEP135 localizes in the proximal lumen of the mother centriole in cells bearing overamplified centrioles induced by Plk4 overexpression (Kleylein-Sohn et al., 2007). The antibody used in their study was raised against the whole C-terminal portion ranging from 649-1140, thus the resolution of the performed assay is rather limited. Nevertheless, those results revealed the localization of HsCEP135 649-1140 in the proximal centriolar lumen. Interestingly, Sonnen et al. found via super-resolution microscopy that HsCEP135 is forming a ring of 170 nm diameter which

Figure 4.2. Human CEP135 and microtubules depicted to scale.
A: Scheme depicting CEP135 in different orientations on microtubules. CEP135 dimers are symbolized by the gray and white structure schemes, respectively. The schemes are in scale with the microtubules. α-tubulin: blue, β-tubulin: green. Potential steric clashes are highlighted in red.
B: Microtubule section generated from a 5.5 Å EM structure (PDB code: 3J6G). Gray: α-tubulin; black: β-tubulin in comparison with the HsCEP135-N model (see Chapter 2.2.). The microtubule binding region of HsCEP135-N is highlighted in red. HsCEP135 spans more than two tubulin dimers in length.
coincides with C-Nap1 at the proximal end of the centriole (Sonnen et al., 2012). This determined diameter fits well with the one determined for glutamylated tubulin of ~ 166 nm in their study.

Based on the given restraints, several models can be envisaged for the arrangement of CEP135 in the centriolar proximal lumen. One would be that CEP135 binds with its N-terminus to either the A, B or C microtubule of a given triplet and spans the whole centriolar lumen with its further domains. The C-terminus would be protruding between two triplets and would cause the 170 nm diameter ring observed by Sonnen et al. (Sonnen et al., 2012). This model would fit well with the approximate length of an almost fully extended CEP135 of 167 nm. In human centrioles, CEP135 only co-localizes shortly from the S/G2 to the G2/M transition with SAS-6 and the cartwheel, before the cartwheel is lost (Sonnen et al., 2012). Therefore, spanning of the proximal lumen would be possible for CEP135 in human centrioles.

In the centrioles and basal bodies of *C. reinhardti* where the cartwheels remain in the proximal lumen of the centriole throughout the cell cycle, it is sterically unlikely that the CEP135 ortholog Bld10p spans the whole lumen. Truncation studies by Matsuura et al. and Hiraki et al. clearly showed that the C-terminal segments of Bld10p contribute to the cartwheel spokes, while the N-terminal domains locate in the pinhead region (Hiraki et al., 2007; Matsuura et al., 2004). Since Bld10p is 1640 amino acids in length, thus larger than human CEP135 (1140 amino acids), it seems likely that backfolding is involved in fitting Bld10p in the narrow space between SAS-6 and the microtubule wall. This would imply that CEP135 and Bld10p adapt different ways of spatial arrangement in the centriolar lumen or that a more complicated folding pattern is causing the observed localization in both cases.

A longitudinal binding of CEP135/Bld10p to centriolar microtubules with the remaining domains roughly in parallel orientation to the centriolar microtubules could be envisaged. While the longer C-terminus of Bld10 could protrude into the centriolar lumen to form part of the cartwheel spoke, the C-terminus of the shorter human CEP135 would rather remain at the same vertical level as the N-terminus. However, also in this case a more complex folding pattern, e.g., backfolding, seems likely to avoid steric clashes with other CEP135/Bld10p dimers. Finding evidence for such a vertical running density in the cryotomography maps of the *Trichonympha* basal body generated by Guichard et al. is challenging (Guichard et al., 2013). Vertically connected density in the pinhead region was suggested to hold CEP135/Bld10p (Hatzopoulos et al., 2013). This would imply a more
complicated fold with a possible asymmetric behavior of the CEP135/Bld10p homodimer. Even so, since *Trichonympha* is not fully sequenced to date, it is not clear whether CEP135 is apparent in this organism. Furthermore, CEP135/Bld10p could have been lost from the *Trichonympha* basal bodies during preparation or averaged out at the later stage of sub-tomogram averaging.

*C. elegans* seems to lack CEP135/Bld10p and also lacks the equivalent of the A-C linker observed in other species between its microtubule singlets. It is tempting to assume that the A-C linker is constituted by the microtubule binding N-terminus of CEP135. However, truncation studies on Bld10p (CEP135 ortholog) in *C. reinhardtii* showed that even with large domains truncated from the N- and C-terminus an A-C linker is still visible between the microtubule triplets in the electron microscopy cross section images (Hiraki et al., 2007).

Extrapolation of data from different organisms is necessary since no comprehensive set of information is available for a single organism. This is not without pitfalls. Clear differences in sequence and localization indicate possible divergence concerning their mode of action. Furthermore, all mentioned techniques to derive protein localization information have similar disadvantages, such as the epitope accessibility in context of the centriole and the specificity of the antibody. Therefore, in order to distinguish between the different possible arrangements discussed in this section, further experiments are necessary on human CEP135.

Determination of the general in solution structure of the full length CEP135 would provide a strong basis for further deduction of the arrangement of CEP135 in the centriolar lumen. Small angle X-ray scattering of human CEP135 full length or a suitable close ortholog could provide such a low resolution structural information. Nevertheless, a homogeneous, stable, non-aggregating sample is required for SAXS, which proofs to be challenging for such a large protein. In addition, due to the large size expected for the CEP135 dimer a long flight tube will be necessary to recover vital low resolution information, which would otherwise be lost due to the beamstop. Another possible method is rotary metal shadowing electron microscopy, which proved to be a suitable tool for the visualization of coiled-coils (Kitagawa et al., 2011c).

To improve our understanding of the localization, studies using immunogold labeling electron microscopy or super-resolution microscopy with antibodies raised against specific CEP135 domains are necessary. Antibodies raised against the N-terminal domain 1-158 or against the far C-terminus would allow for a localization fine tuning. Preferably, the
Localization studies are conducted for the same organisms as the structural studies. Detailed analysis of structural effects and positioning of truncated domains could be achieved by comparing resin embedded cross sections via electron microscopy to those of wild-type centrioles. A similar approach has been used for Bld10p (Hiraki et al., 2007). This approach requires the depletion of endogenous CEP135 by RNAi or inducible CRISPR-Cas9 systems and transient expression of various truncation constructs in cells.

The question on how CEP135/Bld10p is arranged in the centriolar lumen remains one of the most important ones to be investigated. A full structural understanding of CEP135/Bld10p would provide us with the means to judge whether the assumed function and position of CEPP135/Bld10 is physically possible in the way it is perceived at the moment.

4.4. On the role of SAS-6 and CEP135 in the centriole

Previous structural studies supported the notion that SAS-6 is a major factor to determine and impose the 9-fold symmetry of the centriole and therefore its function is to provide a rigid structural framework to build the 9-fold symmetric microtubule wall (Cottee et al., 2015; Hilbert et al., 2013; Kitagawa et al., 2011c; van Breugel et al., 2014, 2011). However, our results (see Chapter 3) clearly indicated the contribution of additional key proteins to the construction of the 9-fold symmetry. Although a system was designed that allowed the tuning of the SAS-6 cartwheel symmetry via point mutations in vitro, these mutations had surprisingly low impact in C. reinhardtii and human cells. Only disconnecting the microtubule wall and the cartwheel by truncating Bld10p allowed the smaller symmetry expected of the NN24 mutant to take effect on the cartwheel (Chapter 3). The Bld10p NN24 double mutants in C. reinhardtii (bld10bld12::NN24ΔN3 and bld10bld12::NN24ΔC2) displayed a higher symmetry for the microtubule triplets than for the cartwheel spokes indicating that under normal conditions the microtubule wall in turn is restraining the cartwheel spoke symmetry (Hilbert et al., 2016). Furthermore, mutations comparable to NN24 in human cells showed even less alteration in the cartwheel symmetry than in C. reinhardtii. As discussed in Chapter 3 an additional, yet cartwheel-interdependent, mechanism based on the microtubule wall seems to significantly contribute to the centriole symmetry and build up. This poses the question which proteins are involved in this mechanism and what is the exact role of SAS-6.

The additional symmetry restraints from this new mechanism could be based on spacer proteins such as the A-C linker, which connects neighboring microtubule triplets
(Chapter 1.2.). Thus, by imposing a certain circumference via connecting the triplets in a distinct angle, a 9-fold symmetry could be generated. This could be tested using *C. elegans* as a model system. *C. elegans* centrioles possess only microtubule singlets and are lacking the A-C linker. If indeed the A-C linker contributes significantly to the maintenance of the 9-fold symmetry, the use of ceSAS-6 mutants comparable to NN24 should have a strong effect on centriolar symmetry. Further investigations would be needed to clarify which proteins contribute to the A-C linker.

Other possibilities in *C. reinhardtii* would include the involvement of further proteins located at the microtubule wall such as Bld10p. Due to its position at the pinhead close to the centriolar microtubules, the Bld10p N-terminus could potentially bind to microtubules. Furthermore, because of its dimeric nature or further interaction partners, Bld10p could link and stabilize assemblies of microtubule triplets. Thereby, Bld10p could impose 9-fold symmetry. Accordingly, removing the N-terminal part of Bld10p in context of the NN24 Bld12p/SAS-6 mutation (*bld10bld12::NN24ΔN3*) would result in a distorted microtubule wall symmetry. C-terminal truncation (*bld10bld12::NN24ΔC2*) on the other hand should result in breakage of the spoke-microtubule link in the pinhead region and thereby allow different symmetries for cartwheel and microtubule wall (Hilbert et al., 2016). However, both truncations show a similar phenotype (Hilbert et al., 2016) of intact microtubule wall symmetry, while the cartwheel symmetry is altered by the NN24 SAS-6 mutations. This suggests that Bld10p is not central to the unknown symmetry mechanism, but rather important for mediating its effects on the cartwheel.

The presence of the additional symmetry imposing mechanism leads to the question, to which degree SAS-6 and the cartwheel mediate the 9-fold symmetry and have the function that was previously proposed (reviewed in Hirono, 2014). It is possible that two redundant mechanisms, the one based on the cartwheel mainly generated by SAS-6 and the one based on the centriolar microtubule wall with yet unknown components, impose the 9-fold symmetry. According to the present results, it seems that the latter mechanism is able to override the symmetry imposed by the cartwheel. In many cases the 9-fold symmetry of the microtubule wall and the cartwheel was maintained in context of the SAS-6 NN-mutants. Only physical separation of the cartwheel from the microtubule wall via Bld10p truncations resulted in considerably altered cartwheel symmetry. Whether the SAS-6 cartwheel based symmetry restraints would be able to equally override erroneous symmetry information of the second mechanism needs to be investigated. However, the components of the second system first need to be identified before mutational studies can
Furthermore, the progression of the centriole duplication process may play an important role for the influence of the mechanisms. If so, the cartwheel symmetry would dominate the first stages of procentriole formation due to the early recruitment of SAS-6, while the centriole wall-dependent mechanism would dominate later stages. Especially in human cells, where the SAS-6 cartwheel is lost during mitosis (Strnad et al., 2007), such a mechanism of shared symmetry mediation is daunting.

The question remains whether SAS-6 and the cartwheel indeed function as a structural scaffold for the centriole. While a lack of SAS-6 impairs centriole formation in human (Strnad et al., 2007) and greatly reduces the number of normal centrioles in *C. reinhardtii* (Nakazawa et al., 2007), it seems that an altered symmetry of the human SAS-6-based cartwheel rather has an effect on the centriole length than on its symmetry (Hilbert et al., 2016). Therefore, the SAS-6-based cartwheel may not be a fixed structural scaffold, which provides rigid and stabilizing connections, but rather a 9-fold symmetric hub. This hub would ensure the recruitment and symmetric distribution of downstream acting proteins to 9-fold symmetric positions along the cartwheel spokes. This hub is then later replaced in human cells by CEP135, which stabilizes the microtubule wall and acts as a hub itself for a further set of proteins, such as C-Nap1 (Kim et al., 2008), that are important for later stages of the centriole duplication cycle. The reduced centriolar length could be linked to the reduced number of SAS-6 cartwheel spokes by a diminished number of CPAP recruited via STIL, which in turn forms a complex with SAS-6 (reviewed in Gönczy, 2012). However, in *C. reinhardtii* the NN24 mutant effected the centriolar symmetry and steered towards 9-and 8-fold compared to pure 9-fold symmetry for the investigated wild-type cells. This indicates that SAS-6 influences the centriolar symmetry at least to a certain degree.

Closely linked to the potential role of SAS-6 is that of CEP135/Bld10p. *Chlamydomonas, Paramecium, and Tetrahymena* CEP135/Bld10p resides at the spoke region of the cartwheel and seems to be the connecting link between the microtubule wall and the cartwheel (Bayless et al., 2012; Hiraki et al., 2007; Jerka-Dziadosz et al., 2010). In addition, Bld10p contributes to the outer parts of the cartwheel spokes (Hiraki et al., 2007; Matsuura et al., 2004). Due to the described phenotypes in the literature it is therefore not only the link between the proposed interdependent mechanisms for centriole symmetry, but it also stabilizes the centriolar microtubules (reviewed in Hirono, 2014). Furthermore, it might be a recruiting hub in itself interacting with various interaction partners and thereby
acting synergistically with SAS-6.

In the case of human CEP135, we located the microtubule binding site in the N-terminal coiled-coil domain of CEP135. Due to its confirmed microtubule binding activity it is likely that human CEP135 plays a major role in stabilizing centriolar microtubules and acts as a hub for its interaction partners such as C-Nap1, CPAP, P50-dynactin (Kim et al., 2008; Lin et al., 2013; Uetake et al., 2004; Yang et al., 2006). Interestingly, human cells do not maintain the cartwheel and a weak co-localization of CEP135 and SAS-6 was only found from the S/G2 transition to the late G2/M phase (Sonnen et al., 2012). In agreement with this observation, human SAS-6 does not depend on human CEP135 for microtubule binding according to a recent study by Gupta et al. describing a C-terminal microtubule binding domain of SAS-6 (Gupta et al., 2015). Therefore, it is unlikely that human CEP135 has the same bridging function as in other organisms like C. reinhardtii, although it was found that human CEP135 contains a SAS-6 binding site (Lin et al., 2013).

In human cells SAS-6 is exchanged for CEP135 during the G2/M phase, once it fulfilled its purpose as a symmetry imposing hub. So instead of acting in union with human SAS-6, as Bld10p does with Bld12p in C. reinhardtii, human CEP135 acts in succession to SAS-6. Thereby, one potentially stabilizing hub protein, SAS-6, which acts as a platform for proteins important for procentriole formation, is replaced by another stabilizing hub protein. This other hub protein, CEP135, interacts with additional proteins important for the later stages of centriole formation and mature daughter centrioles. For instance, C-Nap1 which acts in establishing the linker between mother and daughter centriole. This would explain why in human cells and in vertebrates in general CEP135 seems to be redundant for procentriole formation, but is essential for the integrity of the centriole (Lalor et al., 2013; Tang et al., 2011). The described ability to bind to CPAP appears to be significant, since depletion of CEP135 in human and Drosophila cells lead to shortened centrioles (Blachon et al., 2009; Lin et al., 2013; Mottier-Pavie and Megraw, 2009). Furthermore, human CEP135 might fulfill additional functions. Drosophila CEP135 is crucial in establishing the central microtubule pair in cilia and flagella (Carvalho-Santos et al., 2012). Both orthologs are similar in domain organization, localization and both contain a microtubule binding N-terminal domain (see Chapter 1 and 2). Therefore, human CEP135 may likewise be important for establishing the central microtubule pair in cilia and flagella.
4.5 Conclusion

The function of CEP135/Bld10p in the centriole seems to be the stabilization of the centriole wall by interactions with microtubule multiplets in the proximal luminal site, while likewise providing a platform for further proteins which are important for centriole elongation or linkage. Whether or not CEP135 forms the link between the cartwheel and the centriolar microtubules and to which degree and at what stage it contributes to the procentriole formation appears to be organism dependent. Therefore, pursuing functional studies in different model organisms is important to reveal potential differences in the mode of action and to verify to which degree Bld10p and CEP135 from different species are indeed true functional orthologs.

The role of SAS-6 is the formation of the cartwheel hub and major parts of the cartwheel spokes, which influences to a certain extend the 9-fold symmetry. In addition, downstream acting proteins are recruited to the 9-fold symmetric positions a the spoke tips using the cartwheel as a guiding hub via direct and indirect interactions. Later the second interdependent mechanism supports the 9-fold symmetry and is partially able to correct for improper symmetry imposed by SAS-6. How and to which extend this two mechanisms influence the centriolar architecture requires further investigation. A special focus should be on the role of CEP135/Bld10p as the apparent connecting link between the two mechanisms and how this translates to the human centriole where the interaction of SAS-6 and CEP135 seems to be of less importance.
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Appendix

Introductory note

Besides the reported projects, contributions to several other crystallographic projects were made. The contributions focused on phasing and refinement of challenging datasets as well as on the analysis of complementing SAXS data. The methods, results and discussion sections described herein are limited to the contribution by the author.

A.1. The antiparallel coiled-coil of Kif21a

INTRODUCTION

The following abstract is an excerpt from the manuscript with the title “Structural basis for misregulation of kinesin KIF21A autoinhibition by CFEOM1 disease mutations” by the authors: Sarah Bianchi, Wilhelmina E. van Riel, Sebastian H. W. Kraatz, Natacha Olieric, Daniel Frey, Eugene A. Katrukha, Rolf Jaussi, John Missimer, Ilya Grigoriev, Vincent Olieric, Roger M. Benoit, Michel O. Steinmetz, Anna Akhmanova, Richard A. Kammerer (see publication list).

“Tight regulation of kinesin activity is crucial, and motor malfunction is linked to neurological diseases and cancer. Point mutations in the KIF21A gene cause congenital fibrosis of the extraocular muscles type 1 (CFEOM1) by disrupting the autoinhibitory interaction between the KIF21A motor domain and a regulatory region in the coiled-coil stalk. However, the molecular mechanism underlying the misregulation of KIF21A activity in CFEOM1 is not understood. Using a multidisciplinary approach, we here show that the regulatory domain of KIF21A containing all disease-associated amino acids found in the stalk domain, folds into an intramolecular antiparallel coiled-coil that inhibits the kinesin through a direct interaction with its motor domain. CFEOM1 mutations lead to hyperactivation of KIF21A by affecting either the structural integrity of the antiparallel coiled-coil or the binding interface, thereby reducing its affinity for the motor domain. Direct interaction of the KIF21A regulatory domain with the related KIF21B motor and sequence similarities to KIF7 and KIF27 strongly suggest a conservation of this regulatory mechanism in other kinesin-4 family members.”
METHODS

Phasing and refinement of the antiparallel coiled-coil crystal structure
In order to reveal the structure and to confirm the predicted monomeric antiparallel coiled-coil, structure determination was attempted by crystallography. The corresponding construct CC1 (938-1017) was purified and crystallized by S. Bianchi (Paul Scherrer Institut). Data was acquired by S. Bianchi (Paul Scherrer Institut) and Dr. V. Olieric (Paul Scherrer Institut) from a single crystal at the X06DA beamline of the Swiss Light Source at 100 K to the resolution of 2.5 Å using a wavelength of 2.066 Å. Due to the high sulfur content Sulfur-SAD phasing was initially attempted by S. Bianchi (Paul Scherrer Institut) and Dr. V. Olieric (Paul Scherrer Institut). However, after experimental phasing failed, computational phasing was performed by the author with support from Dr. M. Wang (Paul Scherrer Institut). Data reduction, merging, scaling and format conversion were done with the programs XDS, XSCALE and XDSCONV (Kabsch, 2010a, 2010b). An advanced molecular replacement strategy was employed using the program AMPLE (Bibby et al., 2012). Briefly, this pipeline prepares and forwards a large number of decoy structures from a ROSETTA (Kaufmann et al., 2010; Rohl et al., 2004) ab initio prediction to PHASER (McCoy et al., 2007). The necessary 3-mer and 9-mer amino acid fragments were generated using the ROBETTA web server (Kim et al., 2004). Automated model building is then attempted after promising solutions have been identified by chain tracing via SHELXE (Thorn and Sheldrick, 2013).

The initial model from AMPLE was subjected to several rounds of manual and automated model building using the programs COOT (Emsley et al., 2010) and BUCCANEER (Cowtan, 2006). In general, refinement was performed using Phenix.refine from the PHENIX suite (Adams et al., 2010) and COOT for rounds of manual refinement. Despite the initially suggested space group of P622 (identified with XTRIAGE the stalled refinement Rwork/Rfree (0.26/0.32) as well as clashing symmetry mates suggested twinning. The structure was built and refined in the space group P3121 applying the twin law -h,-k, l. This allowed a direct refinement of the dimeric interface. The final evaluation of the model was performed with MOLPROBITY (Adams et al., 2010). The model deposited in the PDBe (accession code: 5D3A) yielded a final Rwork/Rfree of 0.1770/0.2034.
Small angle X-ray scattering measurements on the Kif21a antiparallel coiled-coil.

In order to verify whether the anti parallel coiled-coil of Kif21 is indeed monomeric, low resolution structural information of the shape in solution was required. SAXS data collection was performed by S. Bianchi (Paul Scherrer Institut) at the cSAXS (X12SA) beamline at the Swiss Light Source (Paul Scherrer Institut). Data was acquired as previously described (Kisko et al., 2011). Initial data integration and scaling was performed by Dr. J. Missimer (Paul Scherrer Institut). The data was further analyzed by the author using the ATSAS software suite (Chen et al., 2010). The buffer subtracted curves were cut and merged with PRIMUS (Konarev et al., 2003). The distance distribution function was derived from the experimental data using GNOM (Svergun, 1992). The GNOM output was further used as input for the ab initio shape reconstruction with the programs DAMMIF/DAMMIN using the interactive mode (Franke and Svergun, 2009). DAMMIF was used to generate 10 initial reconstructions, which were averaged and filtered with DAMAVER (Volkov and Svergun, 2003). The filtered average was subsequently used as an input model for 10 further refinement runs with DAMMIN. The best refined envelope was identified by the lowest NSD value (normalized spatial discrepancy) to the most typical envelope from the DAMMIF run (which in turn is the one with the lowest NSD to the models considered for averaging).

To compare the obtained results to those expected for the dimeric crystal structure and for the monomeric antiparallel coiled-coil (model generated by S. Bianchi) theoretical scattering curves were computed for both with CRYSOL (Svergun et al., 1995). Subsequently, the distance distribution functions were derived from the theoretical scattering curves using GNOM.
RESULTS AND DISCUSSION

The final crystal structure (for statistics see Table A1.1.) revealed a dimeric antiparallel coiled-coil in the asymmetric unit (see Figure A1.1.A), despite the expected monomeric antiparallel coiled-coil (Figure A1.1.B). The interface of the predicted antiparallel coiled-coil is preserved in the crystal structure. The loop region seemed to allow an opening of the monomeric antiparallel coiled-coil which resulted in a domain swap, thereby creating an antiparallel coiled-coil dimer. However, the comparison of the SAXS measurements with the theoretical distance distribution functions for a dimeric antiparallel coiled-coil and a monomeric antiparallel coiled-coil clearly showed that in solution a monomeric antiparallel conformation is apparent (Figure A1.1.C). Therefore, it is safe to assume that the dimeric conformation observed in the crystal structure was likely a crystal artifact.

Figure A1.1. Crystal structure and SAXS data analysis
(A) Crystal structure of the Kif21a antiparallel coiled-coil
(B) Predicted model of the antiparallel monomeric coiled-coil generated with I-TASSER (courtesy of S. Bianchi) (Zhang, 2008).
(C) Theoretical distance distribution functions of the Kif21a antiparallel coiled-coil crystal structure (black) and the predicted monomeric model (Figure 1B) (red) compared to the distance distribution function derived from experimental SAXS data on the Kif21a antiparallel coiled coil (gray).
(D) Monomeric model fitted to the best envelope from the refined shape reconstruction based on the acquired Kif21a antiparallel coiled coil SAXS data.
Table A1.1. Data collection and refinement statistics.

<table>
<thead>
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<th>Data collection</th>
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<tr>
<td><strong>Wavelength</strong></td>
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<td>Resolution range</td>
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<tr>
<td>Space group</td>
<td>P 31 2 1</td>
</tr>
<tr>
<td>Unit cell</td>
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<td>Total reflections</td>
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</tr>
<tr>
<td>Unique reflections</td>
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<tr>
<td>Multiplicity</td>
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<tr>
<td>Completeness (%)</td>
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<tr>
<td>Mean I/σ(I)</td>
<td>15.84 (1.40)</td>
</tr>
<tr>
<td>Wilson B-factor</td>
<td>48.3</td>
</tr>
<tr>
<td>R-merge</td>
<td>0.1762 (1.785)</td>
</tr>
<tr>
<td>R-meas</td>
<td>0.1814 (1.847)</td>
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<tr>
<td>CC1/2</td>
<td>0.999 (0.472)</td>
</tr>
<tr>
<td>CC*</td>
<td>1 (0.801)</td>
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<table>
<thead>
<tr>
<th>Data refinement</th>
<th></th>
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<tr>
<td>Reflections used in refinement</td>
<td>18494** (972)</td>
</tr>
<tr>
<td>Reflections used for R-free</td>
<td>493 (49)</td>
</tr>
<tr>
<td>Twin law</td>
<td>-h,-k,l</td>
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<tr>
<td>Estimated twin fraction</td>
<td>0.479</td>
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<tr>
<td>R-work</td>
<td>0.1770 (0.2598)</td>
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<tr>
<td>R-free</td>
<td>0.2034 (0.3580)</td>
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<td>CC(work)</td>
<td>0.954 (0.601)</td>
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<td>CC(free)</td>
<td>0.970 (0.477)</td>
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<td>Number of non-hydrogen atoms</td>
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<td>macromolecules</td>
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<td>ligands</td>
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<td>Protein residues</td>
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<td>Ramachandran favored (%)</td>
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<td>Ramachandran allowed (%)</td>
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<td>macromolecules</td>
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<td>ligands</td>
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<td>solvent</td>
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Statistics for the highest-resolution shell are shown in parentheses. Friedel mates were averaged when calculating reflection statistics.

** Friedel mates were kept separate during refinement.
A.2. The TOG domain of CEP104

CEP104 (centrosomal protein of 104 kDa) is a centrosomal protein that is localized at the distal end of the mother centriole and to the tip of the growing flagella. It was suggested to be implicated in cilia formation (Satish Tammana et al., 2013). Among different domains it comprises a section with high sequence similarity to the microtubule binding TOG domains (Tumor Overexpressed Gene).

METHODS

The TOG domain of CEP104 was crystallized by Dr. L Rezabkova (Paul Scherrer Institute). Native and selenomethionine SAD data were acquired by Dr. G. Capitani (Paul Scherrer Institute) and Dr. L. Rezabkova at the X06SA beamline of the Swiss Light Source to a resolution of 1.4 Å and 2.10 Å, respectively. The structure was solved in the space group P22121 using AUTOSOL (Terwilliger et al., 2009). The initial model building was done via AUTOBUILD and was taken as molecular replacement search model for phasing the native dataset by PHASER (McCoy et al., 2007). Preliminary manual model building and refinement were performed via COOT (Emsley et al., 2010) and Phenix.refine from the PHENIX suite (Adams et al., 2010).

RESULTS

TOG crystal structure consists of 1 monomers per asymmetric (Figure A.2.1. A) unit and displays the typical TOG domain fold (Figure A.2.1. B, aligned using PDBsFold (Krissinel and Henrick, 2003). Initial refinement resulted in the statistics described in the preliminary crystallographic table (Table A.2.1.).
Figure A.2.1. CEP104 TOG domain - crystal structure and alignment
(A) Crystal structure of the CEP104 TOG domain (gray). Displayed is the content of the asymmetric unit. (B) Crystal structure of CEP104 TOG domain (gray) aligned to the most similar structure identified by PDBFold: TOG2 domain of Crescerin 1 from Mus musculus (black, PDB ID: 5DN7:A). Q-score (quality score): 0.31, RMSD: 2.94 Å, sequence identity: 14%
Table A2. Data collection and refinement statistics.

<table>
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<th>Data collection</th>
<th>TOG selenomethionine dataset</th>
<th>TOG native dataset</th>
</tr>
</thead>
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<td><strong>Wavelength</strong></td>
<td>0.96 Å</td>
<td>1.0 Å</td>
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<tr>
<td><strong>Resolution range</strong></td>
<td>42.23 - 2.1 (2.175 - 2.1)</td>
<td>43.83 - 1.4 (1.45 - 1.4)</td>
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<td><strong>Space group</strong></td>
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<td>P21212</td>
</tr>
<tr>
<td><strong>Unit cell</strong></td>
<td>46.15 56.21 104.72 90 90 90</td>
<td>48.42 57.28 103.09 90 90 90</td>
</tr>
<tr>
<td><strong>Total reflections</strong></td>
<td>858343 (88163)</td>
<td>917322 (92415)</td>
</tr>
<tr>
<td><strong>Unique reflections</strong></td>
<td>16513 (1607)</td>
<td>55850 (5607)</td>
</tr>
<tr>
<td><strong>Multiplicity</strong></td>
<td>52.0 (54.8)</td>
<td>16.4 (16.5)</td>
</tr>
<tr>
<td><strong>Completeness (%)</strong></td>
<td>1.00 (1.00)</td>
<td>0.97 (1.00)</td>
</tr>
<tr>
<td><strong>Mean I/sigma(I)</strong></td>
<td>33.54 (2.30)</td>
<td>18.98 (1.10)</td>
</tr>
<tr>
<td><strong>Wilson B-factor</strong></td>
<td>42.43</td>
<td>21.43</td>
</tr>
<tr>
<td><strong>R-merge</strong></td>
<td>0.1347 (2.472)</td>
<td>0.06518 (2.66)</td>
</tr>
<tr>
<td><strong>R-meas</strong></td>
<td>0.136 (2.494)</td>
<td>0.06726 (2.744)</td>
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<tr>
<td><strong>CC1/2</strong></td>
<td>1 (0.754)</td>
<td>1 (0.45)</td>
</tr>
<tr>
<td><strong>CC</strong></td>
<td>1 (0.927)</td>
<td>1 (0.788)</td>
</tr>
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</table>

**Data refinement**

| Reflections used in refinement         | 55785 (5607)                 |
| Reflections used for R-free           | 1995 (201)                   |
| R-work                                 | 0.2216 (0.3877)              |
| R-free                                 | 0.2481 (0.3865)              |
| CC(work)                               | 0.962 (0.698)                |
| CC(free)                               | 0.943 (0.714)                |
| Number of non-hydrogen atoms          | 2393                         |
| macromolecules                         | 2082                         |
| Protein residues                       | 258                          |
| RMS(bonds)                             | 0.004                        |
| RMS(angles)                            | 0.86                         |
| Ramachandran favored (%)              | 99                           |
| Ramachandran allowed (%)              | 0.76                         |
| Ramachandran outliers (%)             | 0                            |
| Rotamer outliers (%)                  | 1.7                          |
| Clashscore                             | 3.98                         |
| Average B-factor                      | 30.32                        |
| macromolecules                         | 28.87                        |
| solvent                                | 40.07                        |

*Statistics for the highest-resolution shell are shown in parentheses.
### A.3. List of deposited structures

<table>
<thead>
<tr>
<th>Title</th>
<th>PDB ID</th>
<th>Authors</th>
<th>Status</th>
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<tbody>
<tr>
<td>N-terminal domain of C. Reinhardtii SAS-6 homolog bld12p F145W (NN2)</td>
<td>4TPZ</td>
<td>Hilbert, M., Kraatz, S.H.W.</td>
<td>released</td>
</tr>
<tr>
<td>N-terminal domain of C. Reinhardtii SAS-6 homolog bld12p Q93E (NN10)</td>
<td>4TQ7</td>
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<td>released</td>
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<tr>
<td>N-terminal domain of C. Reinhardtii SAS-6 homolog bld12p K105C F145C (NN18)</td>
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<td>N-terminal domain of C. Reinhardtii SAS-6 homolog bld12p variant G94C K146C (NN19)</td>
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<tr>
<td>N-terminal domain of C. Reinhardtii SAS-6 homolog bld12p Q93E G94D K146R Q147R (NN23)</td>
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<td>released</td>
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<td>Hilbert, M., Kraatz, S.H.W.</td>
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<td>CrBld10-N 1-70</td>
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<td>CEP104 TOG domain</td>
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<td>Rezabkova, L., Kraatz, S.H.W.</td>
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</tr>
<tr>
<td>KIF21A regulatory coiled coil</td>
<td>5D3A</td>
<td>Bianchi, S., Kraatz, S., Steinmetz, M.O., Kammerer, A.R.</td>
<td>released</td>
</tr>
</tbody>
</table>
A.4. Curriculum vitae

Personal Details
Name: Sebastian Kraatz
Address: Kirchstrasse 1, 8953 Dietikon, Switzerland
Nationality: German
Date of birth: 4 June 1988, Munich

Education
Jan. 2012 to now  Ph.D. studies in Structural Biology at the Eidgenössische Technische Hochschule Zürich (ETHZ), Switzerland, Paul Scherrer Institute, Laboratory for Biomolecular Research
Expected thesis defense date: 03/03/2016
Preliminary Thesis title: ‘Structural investigations into functional aspects of the centriolar key proteins CEP135 and SAS-6’
Thesis supervisor: Prof. Michel O. Steinmetz, Prof. G. Schertler

Oct. 2010 – Sep. 2011 Master of Research in Structural Molecular Biology at Imperial College London, U.K., Faculty of Natural Sciences
Title of 1st Thesis: ‘Structural characterization of the interaction between human RNF4 E3 ligase and poly-SUMO moieties’
Thesis supervisor: Dr. P. Simpson, Prof. S. J. Matthews
Title of 2nd Thesis: ‘Evaluation of a novel scaffold structure for enzyme design’
Thesis supervisor: Dr. J. W. Murray
Final Grade: Distinction

Oct. 2007 – Sep. 2010 B.Sc. in Molecular Life Sciences at the University of Hamburg, Germany, Department of Chemistry
Thesis title: ‘Characterisation of hypericin as an inhibitor of the P. falciparum Glutathione-S-transferase’
Thesis supervisor: Dr. M. Perbandt, Prof. C. Betzel
Final grade: 1,24

1998 to 2007 German A-Level, Alexander-von-Humboldt secondary school, Hamburg, Germany
Final grade: 1.2

Scholarships and Awards
2011 1 year Imperial-DAAD Exchange Scholarship
2011 1 year DAAD scholarship

2010 Award for the best bachelor degree in Molecular Life Sciences; awarded by the Sponsor Society of the Department of Chemistry

**Professional Experience**

**Conferences/Retreats**

Biomolecular Structure and Mechanism graduate school retreat 2015 (poster presentation)
EMBO Conference Series: Centrosomes and spindle pole bodies 2014 (poster presentation)
Biomolecular Structure and Mechanism graduate school retreat 2014 (talk)
EMBO Conference Series Microtubules: Structure, regulation and functions 2014 (poster presentation)
27th Rhine-Knee Regional Meeting on Biocrystallography 2013
Recent Advances in Macromolecular Crystallisation 2013 (poster presentation)
Biomolecular Structure and Mechanism graduate school retreat 2013 (poster presentation)
Biomolecular Structure and Mechanism graduate school retreat 2012

**Workshops/Summer Schools**

INSTRUCT course on Computational Tools Combining Atomic and Volume Data 2015, Rutherford Appleton Laboratory Diamond Synchrotron, UK
EMBO Practical Course: Solution Scattering from Biological Macromolecules 2014, EMBL Hamburg, Germany
Global Phasing Ltd. Sharp, Autoproc, Buster workshop 2014, Swiss Light Source, Switzerland
International Summer School of Crystallography 2014, DESY Hamburg, Germany
HADDOCK Workshop 2012, ETH Zurich, Switzerland

**Traineeships**

01/08/2009 to 30/09/2009 Industrial placement at the Eppendorf Instrumente GmbH at the branch Anwendungstechnik

2006 One year chemical technical assistant Traineeship (in the afternoon at another secondary school as a preparation for the traineeship as chemical technical assistant).

**Methods**

**Bioinformatics**: Structure prediction with ROSETTA and ITASSER, multiple sequence alignments using CLUSTAL W, molecular docking with HADDOCK

**Molecular biology**: Cloning and mutagenesis, restriction free cloning methods

**Protein biochemistry**: Protein expression in *E. coli*, protein purification
**Biophysics:** Circular dichroism measurements, multi angle light scattering, dynamic light scattering

**Electron Microscopy:** Negative staining and rotary metal shadowing of protein samples, basic data collection at JOEL microscopes

**SAXS:** Data collection, data analysis and ab initio shape reconstruction

**X-ray crystallography:** Various vapor diffusion techniques, manual screening in 24 well plates, automatic screening using Mosquito and Phoenix robots, seeding, streak seeding, crystal fishing and freezing, room temperature data collection, data collection at 100K, molecular replacement, heavy atom based phasing using SAD and MAD, sulfur SAD phasing, AMPLEx for prediction based phasing, refinement with Refmac and Phenix.refine, Phenix and CCP4 suite in general

**Language skills**

German (mother tongue), English (fluent)

**Computer skills**

Windows, Word, Power Point, Excel, CCP4 suite and Coot (good), Pymol (good), Linux (good), HKL2map (good), Rosetta (good), Phenix (good), basic programming skills in R, basic programming skills in C and C++, basic programming skills in Python
A.5. List of publications

Publications based on projects conducted during Ph.D. studies:


Publications based on projects conducted during Master studies:


References


Bibby, J., Keegan, R.M., Mayans, O., Winn, M.D., Rigden, D.J., 2012. AMPL: a cluster-and-

Blachon, S., Cai, X., Roberts, K. a, Yang, K., Polyansovsky, a, Church, a, Avidor-Reiss, T., 2009. A proximal centriole-like structure is present in Drosophila spermatids and can serve as a model to study centriole duplication. Genetics 182, 133–144.


tubulin. Some common factors that affect this process and the assembly of tubulin. FEBS Lett. 73, 147–50.


Yang, J., Adamian, M., Li, T., 2006. Rootletin interacts with C-Nap1 and may function as a physical linker between the pair of centrioles/basal bodies in cells. Mol. Biol. Cell 17, 1033–40.


