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Molecular Mechanisms of Centriole Assembly

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Molly Marie Lettman

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2012
The Dissertation of Molly Marie Lettman is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2012
Dedication

This thesis is dedicated, in loving memory, to my mom, Linda Simon, whose unconditional love and support made me who I am today.
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Chapter 2, in full, has been submitted for publication and is under review at Developmental Cell. It may appear as Molly M. Lettman, Yao Liang Wong, Valeria Viscardi, Sherry Niessen, Sheng-hong Chen, Andrew K. Shiau, Huilin Zhou, Arshad Desai, and Karen Oegema. Direct Binding of SAS-6 to ZYG-1, and Not Its Phosphorylation by ZYG-1, Recruits SAS-6 to the Mother Centriole for Cartwheel Assembly. The dissertation author was the primary researcher and author of this paper.

Chapter 3, in full, is in preparation for submission. It may appear as Molly M. Lettman, Valeria Viscardi, Yao Wong, Arshad Desai, and Karen Oegema. Three Distinct Regions of SAS-6 are Required for its Recruitment to the Mother Centriole. The dissertation author was the primary researcher and author of this paper.
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ABSTRACT OF THE DISSERTATION

Molecular Mechanisms of Centriole Assembly

By

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Doctor of Philosophy in Biomedical Sciences
University of California, San Diego, 2012
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Centrioles function in the recruitment of pericentriolar material proteins to form centrosomes, the main microtubule organizing centers of animal cells, and in templating the formation of cilia, which are important for many sensory, motility, and signaling functions. Centrioles are cylindrical organelles comprised of a structure termed the cartwheel, which contains a ~25 nm central hub and nine-fold symmetric spokes emanating outward, and an outer wall of stabilized microtubules, which are also nine-fold symmetric. Regulation of centriole assembly, such that it occurs precisely once per cell cycle, is critical to maintain the correct number of centrosomes and thus bipolar spindle formation. Aberrant centriole number results in a loss of chromosome segregation fidelity. Additionally, structural defects in centrioles lead to defective cilia and are one of the underlying causes of a class of diseases called ciliopathies. In the last decade, many of the genes involved in centriole
assembly have been identified and the precise roles of these genes in the process of centriole assembly are beginning to be elucidated.

SAS-6 is the main structural component of the centriolar cartwheel. The work presented here uncouples, for the first time, the localization of SAS-6 to the site of new centriole assembly from its incorporation into the cartwheel. Investigation into these processes identified three distinct regions of SAS-6 that are required for its localization to the site of new centriole assembly. Additional studies showed that, contrary to previous reports, SAS-6 is likely not the target of the critical kinase ZYG-1. These discoveries have implications on the mechanisms limiting centriole assembly to once per cell cycle.
Chapter 1. Introduction

1.1 Centriole Function

Centrioles are small cylindrical organelles that serve critical cellular functions (Nigg and Raff, 2009; Bettencourt-Dias et al., 2011). Centrioles template the formation of cilia, which are microtubule based cellular projections essential for a variety of sensory, motility, and signaling processes (Satir and Christensen, 2007; Garcia-Gonzalo and Reiter, 2012). Mutations in centriolar proteins contribute to a class of diseases called cilipoathies, which are caused by defects in cilia assembly or function (Kobayashi and Dynlacht, 2011). Mild defects in cilia function result in human disease phenotypes and severe ciliary defects are incompatible with life (Goetz and Anderson, 2010; Green and Mykytyn, 2010; Hildebrandt et al., 2011; Oh and Katsanis, 2012). Centrioles also recruit a matrix of proteins called pericentriolar material (PCM) to form centrosomes, which are the major microtubule organizing centers of animal cells and are important for an array of cellular processes including mitotic spindle formation, cell migration, and cell polarity (Yamashita, 2009; Azimzadeh and Marshall, 2010; Schatten and Sun, 2010; Bornens, 2012).

Centrioles were present in the last common eukaryotic ancestor, although they have been subsequently lost in several evolutionary branches, notably some fungi and higher plants (Debec et al., 2010; Carvalho-Santos et al., 2011). Although some organisms have adopted mechanisms to undergo mitotic spindle formation and cell division in the absence of centrioles, every
organism that generates cilia builds centrioles, contributing to the notion that this is the dominant evolutionarily conserved function of centrioles (Carvalho-Santos et al., 2010; Hodges et al., 2010).

1.2 Centriole Structure

Centrioles range in size from ~100 to 250 nm in diameter and ~100 to 500 nm in length in different species (Cunha-Ferreira et al., 2009a; Loncarek and Khodjakov, 2009; Azimzadeh and Marshall, 2010; Gönczy, 2012). The dominant striking structural feature of centrioles is their nine-fold symmetric outer wall of stabilized triplet microtubules (Figure 1.1).

Inside the microtubule wall lies a structure called the cartwheel; when viewed end-on, it looks like the inner ring and spokes of a wagon wheel. The inner ring, called the central hub, is ~25 nm in diameter and the spokes emanating from the central hub, like the outer microtubule wall, are nine-fold symmetric (Azimzadeh and Marshall, 2010; Gönczy, 2012). The entire cartwheel, which is ~100 nm in length, is built from a stack of these wagon wheel-like plates, at intervals of ~15-20 nm (Guichard et al., 2012; Gönczy, 2012). In centrioles that are longer than ~100 nm in length, the cartwheel lies at one end of the microtubule wall, called the proximal end, and the microtubule wall extends beyond the length of the cartwheel.

Centrioles also have two sets of projections called appendages at their distal, non-cartwheel, end (Azimzadeh and Marshall, 2010; Nigg and Stearns, 2011; Brito et al., 2012). These appendages are also nine-fold symmetric.
From the proximal to distal end of the centriole, the first set of appendages is termed the sub-distal appendages. These appendages are involved in anchoring microtubules (Piel et al., 2000). The appendages furthest out, the distal appendages, are involved in recruiting the centriole to the plasma membrane for ciliogenesis (Hoyer-Fender, 2010; Kobayashi and Dynlacht, 2011).

Some cell types build modified centrioles that may be correlated to their function. In *C. elegans* and *Drosophila* embryos, centrioles are short and lack appendages (Pelletier et al., 2006; Carvalho-Santos et al., 2011; Delgehyr et al., 2012; Figure 1.1). The microtubule wall does not extend significantly past the cartwheel. Additionally, these centrioles do not have the stereotypical outer wall of microtubule triplets. In *C. elegans* embryos, the outer wall is composed of singlet microtubules and in *Drosophila* embryos, doublet microtubules are built (Debec et al., 2010). These differences could be indicative of differences in centriole function in these cell types. Centrioles in these embryonic cells do not template the formation of cilia and thus may not require the distal centriolar structures, which are required for cilia formation. Additionally, *Drosophila* sperm centrioles are exceedingly long (~2.6 µm, Blachon et al., 2009), which may be important for some aspect of sperm function.

Additionally, previous studies had suggested that *C. elegans* centrioles lack a cartwheel and instead have a cylindrical structure with the same dimensions as the cartwheel, but lacking the central hub and spoke structures (Pelletier et al., 2006). More recent structural studies on centriolar proteins
across evolution (Kitagawa et al., 2011b; van Breugel et al., 2011), as discussed in detail below, suggest that the centriolar structure in *C. elegans* is likely very similar to other organisms, it just has not been well documented *in vivo* due to the challenges of maintaining and finding these structures in EM preparations.

1.3 Centriole Assembly

A typical cell in G1 phase of the cell cycle has one centrosome, which contains two centrioles. One of these centrioles was built at least two cell divisions prior and served as the ‘mother’ centriole in the previous division for its ‘daughter’ centriole, to which it is now orthogonally oriented. Although both of these centrioles will serve as mother centrioles during the current cell cycle, these centrioles are not identical as only the older centriole has appendages at its distal end. The younger of the two centrioles will acquire these appendages as the cell proceeds through the next cell cycle (Guarguaglini et al., 2005; Azimzadeh and Marshall, 2010).

Centriole duplication begins as the two orthogonally oriented centrioles lose this orientation during G1 in a process termed centriole disengagement (Tsou and Stearns, 2006a; Nigg, 2007). Around this time, these centrioles also become linked, with a longer radius of movement, by connecting fibers that will keep them near each other until they need to move to opposite sides of the cell for spindle assembly (Nigg and Stearns, 2011).
Assembly of a new daughter centriole next to each of the two centrioles, now both mother centrioles, begins during S phase when cartwheel assembly is initiated. The cartwheel is the first visible intermediate in centriole assembly and its assembly can proceed even if later steps (formation of the microtubule wall) are blocked. Assembly and elongation of the microtubule wall continues during G2 and mitosis (Dammermann et al., 2008; Azimzadeh and Marshall, 2010) so that at metaphase, the centrosome at each pole of the mitotic spindle again contains two orthogonally oriented centrioles. As the cell proceeds through G2/M, the younger mother centriole gains appendage proteins and structurally defined appendages during the next G1 (Nigg and Stearns, 2011).

1.3.1 Once and only once per cell cycle

Centrioles duplicate precisely once per cell cycle. Although the evolutionarily conserved function of centrioles appears to be in cilia assembly (Carvalho-Santos et al., 2010, 2011; Debec et al., 2010; Hodges et al., 2010; Azimzadeh et al., 2012), and even some organisms that typically use centrioles in spindle assembly can undergo cell divisions that are largely normal in the absence of centrioles or in the presence of too many centrioles (Khodjakov et al., 2000; Godinho et al., 2009), aberrant numbers of centrioles lead to errors in chromosome attachments and aneuploidy (Ganem et al., 2009; Nigg and Raff, 2009; Silkworth et al., 2009; Thompson et al., 2010).
Thus maintaining the correct number of centrioles is imperative to the overall fidelity of mitotic divisions.

In order to ensure that centriole assembly occurs exactly once per cell cycle, this process must be highly regulated and intimately linked to cell cycle cues and cell cycle progression. It is likely that there are multiple levels of input from the cell cycle that feed into the process of centriole assembly. In line with this assumption, different centriolar structures assemble at different points of the cell cycle. Initiation of cartwheel formation occurs around the G1/S transition, the microtubule wall is built and the centriole elongates during G2 and mitosis, and appendages are constructed during the subsequent mitosis and G1 (Hinchcliffe and Sluder, 2001; Azimzadeh and Marshall, 2010; Nigg and Stearns, 2011). The precise mechanisms behind all of these cell cycle links and strategies to prevent overproduction of centrioles are only partially unraveled and are discussed further below.

1.3.2 Genes Required for Centriole Assembly: universal assembly module

The last decade has brought major advances in our understanding of centriole assembly on two fronts. First, many of the genes involved in centriole assembly were identified through a variety of genomic and proteomic approaches (Andersen et al., 2003; Li et al., 2004; Keller et al., 2005; Sönnichsen et al., 2005; Goshima et al., 2007; Dobbelaere et al., 2008).
Second, how this list of genes functions to promote centriole assembly is beginning to be understood.

Despite the presence of centrioles in the last eukaryotic ancestor (Carvalho-Santos et al., 2011), only two genes, SAS-6 and SAS-4, are universally conserved in organisms that build centrioles (Carvalho-Santos et al., 2010; Hodges et al., 2010). Both genes were originally identified in the nematode *C. elegans* and they are thought to play structural roles in the centriolar cartwheel and outer wall, respectively (Kirkham et al., 2003; Leidel and Gönczy, 2003; Dammermann et al., 2004; Leidel et al., 2005).

### 1.3.3 Cartwheel Formation

Centriole assembly can be subdivided into temporally and genetically distinct steps. The first detectable structural intermediate in centriole assembly is the cartwheel. Cartwheel assembly is independent of subsequent steps in centriole assembly as it proceeds normally, even if later events in centriole assembly are blocked.

As the cartwheel assembles around the time of the G1/S transition (Hinchcliffe and Sluder, 2001, 2002), Cdk2 complexed with cyclin E and/or A, whose activity is high at the G1/S transition, was suspected to have a role in regulating the initiation of centriole assembly. Several groups uncovered evidence for this direct link to the cell cycle (Hinchcliffe, 1999; Matsumoto et al., 1999; Meraldi et al., 1999) and further work suggested putative substrates (Okuda et al., 2000; Fisk and Winey, 2001; Chen et al., 2002). Later, however,
knockout mice for both Cdk2 and cyclin E proved to be viable, suggesting that either phosphorylation of Cdk2 substrates is not absolutely required or that other kinases can substitute for the loss of Cdk2 (Berthet et al., 2003; Geng et al., 2003; Ortega et al., 2003; Murray, 2004). The precise cell cycle cues underlying the initiation of centriole assembly linking it to the G1/S transition, and whether cyclin dependent kinases are directly involved, requires further investigation.

The molecular pathway of cartwheel assembly has been best defined in *C. elegans*, where only four genes are known to be essential for this step: SPD-2, ZYG-1, SAS-6, and SAS-5 (Pelletier et al., 2006; Figure 1.2).

The first protein recruited to the site of daughter centriole assembly in *C. elegans* is SPD-2 (Delattre et al., 2006; Pelletier et al., 2006), a gene which functions in both centriole assembly and centrosome maturation in *C. elegans* (Kemp et al., 2004; Pelletier et al., 2004). The role of SPD-2 in cartwheel assembly appears to be in the localization of the kinase ZYG-1 (Delattre et al., 2006; Pelletier et al., 2006). Although the sequence is poorly conserved (Carvalho-Santos et al., 2010; Hodges et al., 2010), ZYG-1 is thought to serve the same function as polo-like kinase 4 (Plk4) in other species. These are the only kinases known to be required for early events in centriole assembly (O’Connell et al., 2001; Bettencourt-Dias et al., 2005; Habedanck et al., 2005) and these kinases are not only required for centriole assembly, but their overexpression is sufficient to drive the assembly of too many centrioles per mother centriole (Habedanck et al., 2005; Rodrigues-Martins et al., 2007a).
The role of SPD-2 in recruiting ZYG-1 to the site of new centriole assembly seems to have been delegated to other proteins in *Drosophila* (Dix and Raff, 2007; Giansanti et al., 2008) where it is only required for PCM assembly. In *Drosophila*, recruitment of the kinase Plk4 is performed by the protein Asterless/Cep152 (Dzhindzhev et al., 2010). In human cells, both the involvement of the human homolog of SPD-2, Cep192, in centriole assembly and the requirement for Asterless/Cep152 in recruiting Plk4 to the site of new centriole assembly are controversial (Gomez-Ferreria et al., 2007; Zhu et al., 2008; Cizmecioglu et al., 2010; Dzhindzhev et al., 2010; Hatch et al., 2010; Slevin et al., 2012), suggesting that SPD-2/Cep192 and Asterless/Cep152 might act together to recruit Plk4, or that additional factors may be involved.

Although the kinase activity of ZYG-1/Plk4 is known to be required for centriole assembly (Lettman et al., Chapter 2, this thesis; O’Connell et al., 2001; Habedanck et al., 2005), the molecular mechanisms underlying this requirement remain unknown. Several substrates of ZYG-1/Plk4 have been proposed (Bonni et al., 2008; Kitagawa et al., 2009; Petrinac et al., 2009; Guderian et al., 2010; Hatch et al., 2010; Puklowski et al., 2011a; Bahtz et al., 2012), but the putative substrate that has gained the most attention is another protein required for cartwheel assembly, SAS-6 (Kitagawa et al., 2009).

In *C. elegans*, ZYG-1 is required for the localization of SAS-6 and SAS-5 to the site of centriole assembly (Lettman et al., Chapter 2, this thesis; Delattre et al., 2006; Pelletier et al., 2006). SAS-6 is conserved throughout all
organisms that build centrioles and is universally required for centriole assembly (Dammermann et al., 2004; Leidel et al., 2005; Nakazawa et al., 2007; Rodrigues-Martins et al., 2007b; Yabe et al., 2007; Culver et al., 2009; Carvalho-Santos et al., 2010; Hodges et al., 2010); SAS-5 is a distant relative of the Ana2/STIL family of proteins, which are all are required for centriole assembly (Delattre et al., 2004; Goshima et al., 2007; Dobbelaere et al., 2008; Kitagawa et al., 2011a; Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012).

Two recent seminal papers provide evidence that SAS-6 is the main structural building block of the centriolar cartwheel and responsible for dictating its nine-fold symmetry (Kitagawa et al., 2011b; van Breugel et al., 2011; Figure 1.3). These papers revealed the structure of the N-terminus of SAS-6 from different species and proposed a model for how its structural properties dictated the nine-fold symmetry of the centriolar cartwheel. SAS-6 family members contain an N-terminal globular domain, a long central coiled coil, and a C-terminus that lacks predicted secondary structure. SAS-6 dimerizes through its long central coiled coil domain and, subsequently, a weaker interaction between the N-termini brings the SAS-6 dimers together. The interaction between the N-termini results in a curved structure that is compatible with the N-termini of nine SAS-6 dimers coming together to form the central hub of the cartwheel. The coiled-coil domains of each of these dimers then radiate outward to form the cartwheel spokes. In this model, the C-termini of SAS-6 are placed at the outer edge of the cartwheel, which is
consistent with immuno-EM using an antibody against a C-terminally tagged SAS-6 in Chlamydomonas (van Breugel et al., 2011). The essentially identical structure of the SAS-6 N-terminus from different organisms, including *C. elegans*, suggests that *C. elegans* do indeed build a cartwheel, although it has not been experimentally documented.

A recent cryo-electron tomography study of the *Trichonympha* centriole further illuminated the structure of the cartwheel (Guichard et al., 2012). It has long been appreciated that, when viewed longitudinally, the cartwheel appears as stacks of plates, suggesting that the SAS-6 assemblies also stack on top of one another. This study revealed that these plates are paired vertically, where the cartwheel spokes are linked together at the outer edge by some unknown connection. One molecule that could comprise the link between the SAS-6 coiled coil spokes is SAS-5/Ana2/STIL. In *C. elegans*, SAS-5 is known to be required for cartwheel assembly (Pelletier et al., 2006) and it binds to the SAS-6 coiled coil (Lettman et al., Chapter 2, this thesis; Leidel et al., 2005; Qiao et al., 2012). Although it has been proposed that both SAS-5 and STIL exhibit dynamic behavior at the centriole (Delattre et al., 2004; Vulprecht et al., 2012), there could be a population that is stably associated with the centriole. Ana2 has also been reported to interact with DSas-6 (Stevens et al., 2010a) and simultaneous overexpression of DSas-6 and Ana2 leads to the formation of tubules in the cytoplasm that look similar to the central hub of the centriolar cartwheel (Stevens et al., 2010b). STIL does not seem to bind directly to
HsSAS-6 (Tang et al., 2011; Arquint et al., 2012), suggesting the functions of these proteins might not be fully conserved.

Finally, the last protein that has been proposed to play a critical role in cartwheel assembly in some organisms is Cep135/Bld10. The presence of the Cep135/Bld10 gene has been proposed to be correlated with the presence of centrioles (Carvalho-Santos et al., 2010, 2011), yet no Cep135/Bld10 homolog has been identified in *C. elegans*. Cep135/Bld10 has shown to localize to the outer tip of the spokes of the centriolar cartwheel (Hiraki et al., 2007), placing it in a prime location for connecting the outer SAS-6 spokes. Yet the absolute requirement for Cep135/Bld10 in centriole assembly is still under dispute (Matsuura et al., 2004; Kleylein-Sohn et al., 2007; Mottier-Pavie and Megraw, 2009; Jerka-dziadosz et al., 2010; Carvalho-Santos et al., 2012; Roque et al., 2012) and the possibility exists that Cep135/Bld10 is exclusively required for stabilizing centrioles in the formation of motile cilia. The lack of Cep135/Bld10 in *C. elegans*, which lack motile cilia, building only sensory cilia in neurons, is in line with this idea. Additionally, the main centriolar defect in the absence of Cep135/Bld10 in *Drosophila* appears to be the loss of the central microtubule pair in motile cilia (Mottier-Pavie and Megraw, 2009; Carvalho-Santos et al., 2012; Roque et al., 2012).

SAS-6 is thought to dictate the nine-fold symmetry of the centriole through the interactions between its N-termini, which drive formation of a curved structure (Kitagawa et al., 2011b; van Breugel et al., 2011). These *in vitro* assemblies were not perfect, however. Some deviated from nine-fold
symmetry and some failed to close completely. Additionally, modeling assemblies of the crystal structures is consistent with either a flat ring or a helix (Cottee et al., 2011). This suggests that other factors might regulate the SAS-6 assembly or reinforce the nine-fold symmetry in vivo. Consistently, in *Chlamydomonas* mutants that lack SAS-6 most cells failed to build a centriole, as expected, yet most of the ones that succeeded retained the nine-fold symmetry (Nakazawa et al., 2007).

Interestingly, examples of centrioles that deviate from the typical nine-fold symmetry exist in nature (Phillips, 1967; Riparbelli et al., 2009, 2010), although the organisms that are known to build centrioles that deviate from nine-fold symmetry also build nine-fold symmetric centrioles. Intriguingly, in some instances, these larger centrioles can even be built near nine-fold symmetric mothers. It will be very interesting to examine the mechanisms mediating this deviation from the nine fold symmetry.

The cartwheel is built first during centriole assembly and its structure dictates the symmetry and location of the outer microtubule wall. After construction of the centriole, however, the cartwheel seems to be dispensable for the maintenance of the centriole because it is lost from mature centrioles in vertebrate cells (Alvey, 1986; Guichard et al., 2010). How and why the cartwheel is lost from mature centrioles in some organisms remains an open question.

### 1.3.4 Microtubule outer wall formation
Assembly of the outer wall of microtubules around the cartwheel requires SAS-4 (Pelletier et al., 2006), also known as CPAP and CENPJ in human cells. SAS-4 begins to accumulate at the centrosome during S phase, concomitant with cartwheel assembly, and its levels continue to increase until mitosis (Dammermann et al., 2008). In human cells, the total SAS-4 protein levels increase throughout the cell cycle until mitosis when there is a steep decline that is proteasome dependent (Tang et al., 2009). In *C. elegans*, SAS-4 at the centriole is in dynamic exchange with cytoplasmic pools until late prophase at which point it becomes stabilized and no longer exchanges with cytoplasmic pools (Dammermann et al., 2008). Stabilization of SAS-4 is likely reflective of completion of its assembly into the outer wall as it occurs at the same cell cycle time point as assembly of the microtubule outer wall (Pelletier et al., 2006) and it requires γ-tubulin and microtubule assembly (Dammermann et al., 2008).

SAS-4 family proteins contain an N-terminal tubulin binding domain, several regions of predicted coiled coils, and a C-terminal domain of unknown function that contains homology to a possible paralog of SAS-4, the TCP10 protein, which was originally identified as having a function in sperm (Schimenti et al., 1988; Cebra-Thomas et al., 1991; Hung et al., 2000, 2004; Carvalho-Santos et al., 2010). The tubulin binding domain is able to both bind tubulin dimers and destabilize microtubules in vitro (Hung et al., 2004). Additionally, an interaction between SAS-4 and γ-tubulin has also been
reported (Hung et al., 2000) and human SAS-4 contains a microtubule binding domain (Hsu et al., 2008).

Addition of the microtubules to the outer wall begins with the addition of nine singlet microtubules. In isolated human centrioles, early in the process of microtubule addition, a cap at the proximal end of the singlet microtubules can be detected (Guichard et al., 2010). This cap is believed to be the γ-tubulin ring complex (γ-TuRC) nucleating the microtubule. The microtubules then grow unidirectionally toward the distal end of the centriole. This is in contrast to microtubule assembly in the C. elegans centriole, in which singlet microtubules do not display a strong preference for directional growth and no cap has been detected (Pelletier et al., 2006). γ-tubulin has been shown to be necessary for centriole assembly in a variety of organisms (Ruiz et al., 1999; Shang, 2002; Dammermann et al., 2004, 2008; Haren et al., 2006), but it is unclear if it is required for nucleation of the centriolar microtubules per se. The doublet and triplet microtubules, which are not fully closed 13 protofilament microtubules, grow off the sides of the singlet microtubules. No γ-TuRC cap has been detected on the doublet and singlet microtubules and they seem to grow bidirectionally (Guichard et al., 2010). Studies in Chlamydomonas and Paramecium have suggested a role for ε- and δ-tubulin in assembly of the doublet and triplet microtubules (Dutcher and Trabuco, 1998; Garreau de Loubresse et al., 2001; Dupuis-Williams et al., 2002; Dutcher et al., 2002). However, mutations in α-tubulin can suppress the defects of δ-tubulin (Fromherz et al., 2004) and no homologs of δ- or ε-tubulin have been identified.
in *Drosophila* (Hodges et al., 2010), despite the assembly of centrioles with triplet microtubules in this species, suggesting that these tubulin isoforms might function in stability of the microtubule triplets, rather than being absolutely required for their formation.

### 1.3.4 Centriole elongation

With the notable exceptions of *C. elegans* and certain *Drosophila* cell types, the outer microtubule wall continues to elongate beyond the length of the cartwheel. In human cells, the microtubule wall is ~70% of its final length by the end of S phase and continues its elongation during G2 (Azimzadeh et al., 2009). How the ultimate length of the centriole is determined and how centriole elongation is linked to the cell cycle remain to be fully unraveled, but several recent studies have begun to shed light on these processes.

Proper centriole elongation requires a balance of SAS-4 and CP110. CP110 was originally identified as a CDK substrate required for centrosome duplication, suggesting this might be one direct connection between the cell cycle and centriole assembly (Chen et al., 2002). CP110 localizes to the distal tip of mature and elongating centrioles (Kleylein-Sohn et al., 2007). Upon depletion of CP110 or its binding partner Cep97, which is required for CP110 stability, the centriolar microtubules continue to extend up to several microns in length (Spektor et al., 2007). Overexpression of SAS-4 results in a similar phenotype (Kohlmaier et al., 2009; Schmidt et al., 2009; Tang et al., 2009). These microtubule extensions sometimes appear as structurally normal
centrioles of extended length and sometimes have splayed microtubule ends (Kohlmaier et al., 2009; Schmidt et al., 2009). Localization of specific markers suggest that these structures are elongated centrioles rather than primary cilia (Schmidt et al., 2009), although removal of CP110 is indeed essential for primary cilia formation (Spektor et al., 2007). Interestingly, proteins that mark the distal end of centrioles localized the same distance away from the proximal centriole end in these extended structures (Schmidt et al., 2009). It has been suggested that CP110 caps the growing microtubules and SAS-4 mediates the addition of tubulin subunits under this CP110 cap. Consistent with this model, CP110 has been shown to interact with a recently identified microtubule plus-end tracking protein (Jiang et al., 2012) and the tubulin binding domain of SAS-4 is required for the formation of these microtubule extensions (Tang et al., 2009).

Consistent with the fact that centrioles in C. elegans do not extend significantly beyond the cartwheel, no CP110 homolog has been identified in C. elegans (Hodges et al., 2010). Drosophila sperm centrioles elongate significantly, to a remarkable ~2.6 µm in length (Blachon et al., 2009), and a CP110 homolog can be found in this organism and localizes to the tips of centrioles (Delgehyr et al., 2012). Surprisingly, in somatic Drosophila cells, CP110 removal leads to shorter centrioles rather than longer (Delgehyr et al., 2012). However, centrioles in somatic Drosophila cells are short; as in C. elegans cells, the centrioles do not elongate significantly past the length of the cartwheel. The phenotypic consequences of CP110 removal have not been
investigated in *Drosophila* sperm, which are the only centrioles in *Drosophila* to elongate significantly.

Several proteins in addition to SAS-4 and CP100 are thought to help regulate centriole length. A protein called Centrobin, which localizes specifically to daughter centrioles, has also been proposed to be required for centriole assembly and centriole elongation (Zou et al., 2005; Gudi et al., 2011). Centrobin binds and stabilizes microtubules *in vitro*, and this activity may be enhanced by phosphorylation (Lee et al., 2010). Centrobin is also required for CP110 localization (Gudi et al., 2011), thus it is a bit paradoxical that it is required for centriolar microtubule elongation. One possibility is that Centrobin is required for stability of the elongating centriole, as has been proposed (Gudi et al., 2011), thus masking the overly long centrioles that usually occur in the absence of centriolar CP110.

Poc1 has also been proposed to be involved in centriole elongation. A Poc1 mutation in *Drosophila* results in sperm centrioles that are short (Blachon et al., 2009). These flies are fertile, drawing into question the necessity for Poc1, yet it is unclear if this is a null mutation as the highly conserved N-terminus could be unaffected. Overexpression of Poc1 in human cells results in extended structures reminiscent of those seen upon SAS-4 overexpression (Keller et al., 2009). Yet, as with many components implicated in centriole elongation, distinguishing between requirements for centriole stability and elongation *per se* is challenging because of similar terminal phenotypes. It has also been suggested that Poc1 is required for the former as Poc1 mutants in
*Tetrahymena* display centrioles with severed microtubules in their outer walls (Pearson et al., 2009).

Ofd1, named for its involvement in the ciliopathy orofacialdigital syndrome (Feather et al., 1997; Ferrante et al., 2001, 2006; Romio et al., 2004), localizes to the distal ends of all centrioles (Singla et al., 2010). In the absence of Ofd1, centriole length becomes de-regulated and centriole length varies to a great degree. Loss of Ofd1 ultimately disrupts cilia assembly, which explains the ciliopathy-related phenotypic outcomes of Ofd1 syndrome patients (Singla et al., 2010).

Finally, in the absence of Poc5, a conserved centrin-binding protein, centrioles do not elongate (Azimzadeh et al., 2009). The loss of Poc5, however, results in a cell cycle arrest prior to when centrioles normally elongate (Rattner and Phillips, 1973; Azimzadeh et al., 2009), drawing into question whether Poc5 is required for cell cycle progression or centriole elongation *per se* (Azimzadeh et al., 2009).

Formation of the microtubule wall and centriole elongation are intimately coupled to cell cycle progression and occur during G2 and mitosis (Rattner and Phillips, 1973; Dammermann et al., 2008; Loncarek et al., 2008; Azimzadeh et al., 2009). Again, how cell cycle cues directly feed into these processes remains unknown, but as stated above, CP110 was originally identified as a Cdk substrate (Chen et al., 2002). Additional input from phosphorylation has been proposed (Chang et al., 2010) and the process of centriole elongation is linked to Plk1 activity specific to G2 phase of the cell.
cycle. (Loncarek et al., 2010). The mechanisms by which these phosphorylation events contribute to centriole elongation, however, are not understood. Additionally, the levels of several of the proteins involved in this process have been shown to be cell cycle regulated (Tang et al., 2009; D’Angiolella et al., 2010).

1.3.5 Appendages and asymmetries

As a cell proceeds though mitosis, cartwheel assembly and formation and elongation of the outer microtubule wall have occurred. Assembly of this centriole is not complete, however, until the following cell cycle when the centriole acquires two sets of appendages, distal (furthest from the cartwheel end of the centriole) and sub-distal, both of which display the nine-fold symmetry of the cartwheel spokes and microtubule wall (Vorobjev and Chentsov, 1982; Paintrand et al., 1992; Hoyer-Fender, 2010; Kobayashi and Dynlacht, 2011; Nigg and Stearns, 2011). Thus, even though a cell contains two centrioles upon cytokinesis, both of which will template the formation of a new daughter centriole as the cell cycle progresses, these mother centrioles are asymmetric as one has yet to acquire its appendages.

The distal appendages are important for anchoring the centriole to the plasma membrane during cilia formation (Hoyer-Fender, 2010; Kobayashi and Dynlacht, 2011). Distal appendages are thought to be equivalent to the transition fibers that have long been described in electron micrographs of cilia (Hoyer-Fender, 2010). Cep164 is specifically required for the formation of the
distal, but not sub-distal, appendages and thus is required for cilia assembly (Graser et al., 2007a).

Although microtubules are nucleated to a similar extent near both mother centrioles, the centrioles show a difference in the ability to anchor microtubules in interphase and this function is attributed, in part, to the sub-distal appendages (Piel et al., 2000). Two proteins that have been proposed to mediate the microtubule anchoring activity of the sub-distal appendages are Cep170 and ninein. Both proteins localize to the sub-distal appendages (Bouckson-Castaing et al., 1996; Mogensen et al., 2000; Guarguaglini et al., 2005). Ninein interacts with γ-tubulin and is thought to anchor microtubule arrays both as part of the sub-distal appendages of the centriole and at other locations in different cell types (Mogensen, 1999; Mogensen et al., 2000; Stillwell et al., 2004; Delgehyr et al., 2005). Cep170 has recently been shown to bind to microtubules (Welburn and Cheeseman, 2012). The sub-distal appendages are thought to disassemble during mitosis (Bornens, 2002; Graser et al., 2007a). One mechanism mediating this disassembly might involve phosphorylation of the C-terminus of Cep170. The C-terminus of Cep170 is required for its localization and is an in vitro substrate of Plk1. Consistently in vivo, Cep170 is heavily phosphorylated and associates with Plk1 during mitosis, (Guarguaglini et al., 2005). Additionally ε-tubulin is thought to play a role in the structure/function of the sub-distal appendages, and its protein levels decrease during mitosis (Chang et al., 2003).
Odf2 localizes to and is required for the formation of both distal and sub-distal appendages (Lange and Gull, 1995; Nakagawa et al., 2001; Ishikawa et al., 2005). A fortuitous separation of function allele of Odf2 resulted in centrioles that built proper distal appendages, but the cilia built from these centrioles lacked basal feet, which are thought to be related to sub-distal appendages (Hoyer-Fender, 2010; Kobayashi and Dynlacht, 2011; Kunimoto et al., 2012). Mice bearing these cilia had phenotypes consistent human ciliopathy phenotypes (Kunimoto et al., 2012). It will be interesting to examine this allele in the future to see if centrioles fail to build sub-distal appendages at all timepoints or if these centrioles build proper sub-distal appendages, but they fail to function or be maintained during ciliogenesis.

Several proteins that regulate centriole length are also required for appendage formation, including Ofd1 and Poc5 (Azimzadeh et al., 2009; Singla et al., 2010), but proper centriole length itself is not required for appendage formation, as appendages still form normally in cells depleted of CP110 or overexpressing SAS-4 (Schmidt et al., 2009), both of which disrupt centriole length control.

1.3.6 Connecting fibers

Around G1 when the centrioles lose their orthogonal orientation, or disengage to begin the next round of assembly, the two centrioles become connected by a network of fibers that keep them nearby (Mardin and Schiebel, 2012). These fibers are thought to be composed of the structural proteins
Rootletin and C-Nap1 (Fry et al., 1998a; Mayor et al., 2000; Bahe et al., 2005; Yang et al., 2006) and involve β-catenin (Bahmanyar et al., 2008). Depletion of either Rootletin or C-Nap1 results in premature centrosome separation (Mayor et al., 2000; Bahe et al., 2005) and mutations in β-catenin linked to human cancers result in increased centrosome distance (Bahmanyar et al., 2008). The connecting fibers must be severed before the cell enters mitosis so that the two centrosomes can move to opposite sides of the cell to build the mitotic spindle. This severing involves the phosphorylation of C-Nap1, Rootletin, and β-catenin by Nek2 kinase (Fry et al., 1998b; Mayor et al., 2002; Faragher and Fry, 2003; Bahe et al., 2005; Bahmanyar et al., 2008), whose activity is opposed by PP1 until mitosis (Mi et al., 2007), when Plk1 relieves this antagonism (Mardin et al., 2011).

More recently, the involvement of Cep68 and CDK5RAP2 in the connecting fiber network has also been proposed (Graser et al., 2007b). Cep68 localization and dynamic behavior is quite similar to that of C-Nap1 and Rootletin, but CDK5RAP2 behaves distinctly (Graser et al., 2007b). Whether these proteins are structural components of the fibrous network and whether they undergo the same Nek2 mediated disassembly requires further study.

1.4 Mechanisms limiting centriole overduplication

Centrioles recruit pericentriolar material to form centrosomes, thus centriole number dictates centrosome number. Therefore, in order to ensure
the formation of a bipolar mitotic spindle with a single centrosome at each pole, it is crucial that centrioles assemble once and only once per cell cycle.

The first limit on the production of too many centrioles per cell cycle, or centriole overduplication, is that the presence of a daughter centriole orientated orthogonally, or engaged, to a mother centriole blocks the formation of a second daughter centriole. This was first demonstrated by cell fusion experiments using cells in G1 and G2, which had unduplicated and duplicated centrioles, respectively. The G1 cells would duplicate their centrioles in a variety of environments, but the G2 cells would not, indicating that the presence of a daughter centriole was an intrinsic block to reduplication (Wong and Stearns, 2003). An elegant set of laser ablation experiments confirmed the idea that the presence of an engaged daughter centriole blocks the formation of another daughter. When daughter centrioles were removed by laser ablation, another single daughter centriole could form (Loncarek et al., 2008). Interestingly, the new daughter centriole that formed after laser ablation did not always form on the same side of the mother centriole where the previous daughter had been located, suggesting that there is not a single defined assembly site on the mother centriole.

The mechanisms underlying disengagement and removal of the block to reduplication are areas of intense research. Both the protease separase and the kinase Plk1 are thought to be involved (Tsou and Stearns, 2006b; Tsou et al., 2009; Loncarek et al., 2010; Nigg and Stearns, 2011; Mardin and Schiebel, 2012). As in the control of sister chromatid separation, cohesion
holds the centriole pair together until it is cleaved by separase (Tsou and Stearns, 2006b; Schöckel et al., 2011). A small isoform of shugoshin, sSgoI, protects cohesion until anaphase (Wang et al., 2008). This elegant re-use of and linkage to mechanisms controlling chromosome separation is insufficient, however, as in the absence of separase, centrioles still separate, albeit at a slower pace (Tsou et al., 2009). The Plk1 pathway is necessary and sufficient as in the absence of Plk1, centrioles fail to disengage and in the presence of ectopic activated Plk1, they separate prematurely (Tsou et al., 2009; Loncarek et al., 2010; Wang et al., 2011). It is not clear, however, what the molecular mechanisms underlying the involvement of Plk1 in centriole disengagement are or to what extent its involvement is intertwined with the function of separase. Plk1 has been proposed to regulate both the presence of shugosin at the direct cleavage of cohesion at centrioles (Wang et al., 2008; Schöckel et al., 2011).

The presence of an engaged daughter centriole blocks reduplication of the mother centriole. It is unclear, however, if it is a physical attachment between the mother and daughter centrioles that prevents the formation of a new daughter or rather the presence of a daughter centriole nearby that somehow blocks the initiation of the formation of new daughter centrioles (Loncarek and Khodjakov, 2009). The latter would also help to explain why the daughter centrioles do not duplicate.

In addition to its role in disengagement, a Plk1 modification is required for centrioles to acquire the ability to recruit pericentriolar material (Wang et
This is particularly interesting in light of the idea suggested by several groups that PCM is involved in centriole assembly (Dammermann et al., 2004, 2008a; Loncarek et al., 2008). The PCM is thought to provide a local pool of centriolar components. One interesting hypothesis is the idea that centriole disengagement *per se* is not required for assembly of a new daughter centriole, but rather as the daughter centriole has not acquired the ability to recruit PCM (Wang et al., 2011), the local concentration of centriolar components is not high enough to seed nucleation of a new centriole (Goehring and Hyman, 2012).

Another mode of regulation of centriole assembly arises from the cell cycle regulation of the total cytoplasmic levels of many centriolar proteins (Strnad et al., 2007; Cunha-Ferreira et al., 2009b; Korzeniewski et al., 2009; Rogers et al., 2009; Guderian et al., 2010; Holland et al., 2010; Puklowski et al., 2011b; Tang et al., 2011; Arquint et al., 2012; Peel et al., 2012). Regulation of centriolar protein levels is key as prevention of cell cycle-mediated degradation or overexpression of several different centriole proteins has been shown to lead to simultaneous production of multiple daughter centrioles on a single mother (Habedanck et al., 2005; Kleylein-Sohn et al., 2007; Peel et al., 2007; Rodrigues-Martins et al., 2007a; Strnad et al., 2007; Stevens et al., 2010a). It is of note that the concentration of centriolar protein in cells is sufficient to build many centrioles as new centrioles assemble after laser ablation (La Terra et al., 2005; Loncarek et al., 2008) and overexpression of a single component can drive the formation of many centrioles (Habedanck
et al., 2005; Kleylein-Sohn et al., 2007), thus the temporal recruitment of centriole proteins to the site of centriole assembly also must be tightly regulated.

Additional unknown mechanisms of blocking overduplication could also exist. Apart from the regulation of total protein levels, there could be regulation of protein activity. Key kinases are involved in the cartwheel assembly and disengagement pathways and regulation of enzymatic activity is another likely mode of preventing overduplication.

1.5 Templated vs. de novo Centriole Assembly

Typically, a new daughter centriole is formed adjacent to a mother centriole strictly once per cell cycle. A mother centriole is not strictly required, however. During ciliogenesis, many centrioles are formed simultaneously around a single mother centriole or in the absence of any mothers (Dawe et al., 2007). Also, if the cytoplasm is devoid of centrioles, centrioles can form de novo. This has been experimentally documented by laser ablation of existing centrioles or by overexpression of centriolar proteins in unfertilized *Drosophila* embryos (La Terra et al., 2005; Rodrigues-Martins et al., 2007a) and occurs in nature in organisms where centrioles are not brought in at fertilization by the sperm, such as in parthenogenic organisms or in mouse embryos which develop centrioles de novo after a few rounds of embryonic divisions (Riparbelli et al., 2010; Courtois et al., 2012).
Thus the mother centriole, rather than a true ‘template’ for the formation of a daughter centriole, seems to be a platform for recruiting the necessary factors and environment for centriole assembly. Consistent with this idea, de novo centriole assembly occurs more slowly than assembly in the vicinity of a mother centriole (Rodrigues-Martins et al., 2007a).

1.6 Relevance of Centriole Structure and Function to Human Disease

Abnormalities in centrioles and centrosomes are linked to two main classes of diseases: cancer and ciliopathies (Nigg and Raff, 2009; Bettencourt-Dias et al., 2011). The mechanisms relating centriole and centrosome dysfunction to these diseases are areas of intense research.

1.6.1 Cancer

Two common hallmarks of cancer cells are aberrations in centrosome and chromosome number. Moreover, these two abnormalities often occur together in cancer cells (Pihan et al., 2003; Giehl et al., 2005). These observations led to the idea that abnormal centrosome number drives aneuploidy through the formation of multipolar spindles. It has been documented, however, that cells containing too many centrosomes do not typically undergo multipolar divisions, but rather cluster their centrosomes so that they ultimately form a bipolar spindle (Ring et al., 1982; Kwon et al., 2008; Godinho et al., 2009). Multiple centrosomes at a single pole have recently been shown to lead to incorrect chromosomal attachments, where a single
chromatid is attached to both poles (Ganem et al., 2009; Nigg and Raff, 2009; Silkworth et al., 2009; Thompson et al., 2010), which can result in aneuploidy. Thus deregulation of centrosome number can cause aneuploidy, but precisely how this aneuploidy contributes to cancer progression is an area of active investigation (Holland and Cleveland, 2012).

Centrosome aberrations are also potentially cancer related in additional ways. Centrosomes and centrioles are important for positioning the mitotic spindle (Hinchcliffe, 2001; Feldman et al., 2007; Rebollo et al., 2007; Yamashita and Fuller, 2008; Azimzadeh and Marshall, 2010; Kitagawa et al., 2011a), which is critical for asymmetric cell divisions that are important during development and differentiation (Yamashita, 2009; Morin and Bellaïche, 2011). Defects in the asymmetric cell divisions of stem cells can be tumorigenic (Caussinus and Gonzalez, 2005; Knoblich, 2010) and centrosome dysfunction has been linked to tumorigeneis through disruption of these asymmetric cell divisions (Basto et al., 2008; Castellanos et al., 2008)

1.6.2 Ciliopathies

Cilia dysfunction results in a broad class of phenotypes including kidney defects, mental retardation, retinal degeneration, infertility, obesity, situs inversus (Hildebrandt et al., 2011). These phenotypes relate to the diverse underlying function of cilia in a variety of cell types (Satir and Christensen, 2007; Oh and Katsanis, 2012). Cilia defects can result in defective cilia
assembly *per se* or from underlying defects in the centrioles used to template cilia formation (Singla et al., 2010).

In summary, understanding more about the basic mechanisms of centriole formation will allow us to better understand how centriole function becomes deregulated and contributes to human disease. The goal of this thesis work is to make inroads into these questions by investigating the regulation of the earliest step of centriole assembly, formation of the centriolar cartwheel.

**ABBREVIATIONS**

PCM = pericentriolar material, Plk4 = polo-like kinase 4, Plk1 = polo-like kinase 1
Figure 1.1. Centriole structure. A daughter centriole forms orthogonally to a mother centriole. Fully mature human centrioles are ~450-500 nm in length, contain a triplet outer microtubule wall, and two sets of appendages at their distal ends (Loncarek and Khodjakov, 2009; Azimzadeh and Marshall, 2010; Gönczy, 2012). C. elegans centrioles lack later steps in centriole assembly as they do not elongate, lack appendages, and have an outer wall of singlet microtubules. This makes C. elegans a good system for studying early steps in centriole assembly.
Figure 1.2. The *C. elegans* centriole assembly pathway. The process of centriole assembly has been best described in *C. elegans*. The first step, construction of the centriolar cartwheel, requires SPD-2, ZYG-1, SAS-6, and SAS-5. The second step, assembly of the outer microtubule wall, requires SAS-4, γ-, α-, and β-tubulin (Pelletier et al., 2006; Delattre et al., 2006; Dammermann et al., 2008).
Figure 1.3. SAS-6 is the main structural component of the centriolar cartwheel. SAS-6 contains a globular N-terminus, a long coiled coil, and a C-terminus that lacks predicted secondary structure. The SAS-6 coiled coil drives dimer formation and SAS-6 dimers further oligomerize via an interaction between the N-termini. This results in a structure that is consistent with SAS-6 building the centriolar cartwheel. The SAS-6 N-termini build the central hub and the coiled coils comprise the cartwheel spokes (Kitagawa et al., 2011b; van Breugel et al., 2011).
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Chapter 2. Direct Binding of SAS-6 to ZYG-1, and Not Its Phosphorylation by ZYG-1, Recruits SAS-6 to the Mother Centriole for Cartwheel Assembly

ABSTRACT

Assembly of SAS-6 dimers to form the centriolar cartwheel is controlled by the ZYG-1/Plk4 kinase. Here we show that ZYG-1 recruits SAS-6 to the mother centriole independently of its kinase activity; kinase activity is subsequently required for cartwheel assembly. We identify a direct interaction between ZYG-1 and the SAS-6 coiled-coil that explains its kinase activity-independent function in SAS-6 recruitment. Perturbing this interaction, or the interaction between an adjacent segment of the SAS-6 coiled-coil and SAS-5, prevented SAS-6 recruitment. SAS-6 mutants with alanine substitutions in a previously described ZYG-1 target site or in 37 other residues either phosphorylated by ZYG-1 in vitro or conserved in closely related nematodes, all supported cartwheel assembly, suggesting that SAS-6 is not the critical ZYG-1 target. We propose that ZYG-1 binding to the SAS-6 coiled-coil recruits the SAS-6—SAS-5 complex to the mother centriole, where a ZYG-1 kinase activity-dependent step, whose target is unlikely to be SAS-6, triggers cartwheel assembly.
INTRODUCTION

Centrioles are small cylindrical organelles that vary in size between species (~200x500 nm in mammalian cells and 100x150 nm in C. elegans; Loncarek and Khodjakov, 2009). The prominent structural features of centrioles include a cartwheel, composed of a central hub ~30 nm in diameter and nine radially symmetric spokes, and an outer wall containing a nine-fold symmetric array of stabilized microtubules (Azimzadeh and Marshall, 2010; Carvalho-Santos et al., 2011). Centrioles serve two primary functions in cells: they direct the formation of cilia, which are important for sensory, motile, and signaling functions, and they recruit pericentriolar material (PCM) to form centrosomes that nucleate and organize microtubules (Azimzadeh and Marshall, 2010; Carvalho-Santos et al., 2011; Nigg and Stearns, 2011). In animal cells, centrioles duplicate once per cell cycle via a process that initiates during S-phase concurrent with DNA replication (Loncarek and Khodjakov, 2009; Azimzadeh and Marshall, 2010; Nigg and Stearns, 2011; Brito et al., 2012). Centriole duplication is tightly regulated to ensure that mitotic cells have precisely two centrosomes, as extra centrosomes lead to aberrant chromosome-microtubule attachments and chromosomal instability (Nigg and Raff, 2009; Thompson et al., 2010).

A core set of proteins required for centriole assembly has been identified. SAS-6 and SAS-4/CPAP, initially discovered through functional genomics approaches in C. elegans (Strnad and Gönczy, 2008), are components of a universally conserved eukaryotic module for centriole
assembly (Carvalho-Santos et al., 2010; Hodges et al., 2010). In *C. elegans*, centriole formation also requires SAS-5 (Dammermann et al., 2004; Delattre et al., 2004), a divergent member of the STIL/Ana2 family of proteins that are essential for centriole assembly in mammals and *Drosophila* (Stevens et al., 2010; Castiel et al., 2011; Tang et al., 2011; Kitagawa et al., 2011b; Arquint et al., 2012; Vulprecht et al., 2012). In metazoans, centriole duplication is controlled by the Polo family kinase Plk4 (Bettencourt-Dias et al., 2005; Habedanck et al., 2005), whose functional analog in *C. elegans* is the kinase ZYG-1 (O’Connell et al., 2001; Carvalho-Santos et al., 2010; Hodges et al., 2010).

Molecular epistasis experiments in *C. elegans* analyzing recruitment to the site of new centriole formation have placed ZYG-1 upstream of SAS-5 and SAS-6; ZYG-1 targets independently of SAS-5 and SAS-6 and is necessary to recruit them to the assembly site (Delattre et al., 2006; Pelletier et al., 2006). SAS-5 and SAS-6 are in turn required to recruit SAS-4 (Dammermann et al., 2004; Delattre et al., 2004; Leidel et al., 2005). Ultrastructural work in *C. elegans* has shown that centriole assembly occurs in two genetically separable steps (Pelletier et al., 2006). In the first step, which occurs during S-phase, the cartwheel (also called the central tube in *C. elegans*) forms adjacent to the parent centriole through a process that requires ZYG-1, SAS-5, and SAS-6 (Pelletier et al., 2006), but not SAS-4. The outer centriole wall, which contains the 9-fold symmetric microtubule array, is formed in a second
step that requires SAS-4 (Pelletier et al., 2006) and mitotic entry (Dammermann et al., 2004).

SAS-6 is a key structural component of the cartwheel across systems (Dammermann et al., 2004; Leidel et al., 2005; Pelletier et al., 2006; Nakazawa et al., 2007; Rodrigues-Martins et al., 2007; Yabe et al., 2007; Culver et al., 2009; Jerka-Dziadosz et al., 2010). SAS-6 family proteins have a conserved architecture with an N-terminal head domain, central coiled-coil, and a C-terminal tail that is predicted to be unstructured. SAS-6 forms a dimer held together by a parallel coiled-coil with its two N-terminal domains facing opposite directions at one end (van Breugel et al., 2011; Kitagawa et al., 2011c). Crystal structures revealed that the SAS-6 N-terminal domains interact between dimers via a peg-into-hole mechanism involving insertion of a critical hydrophobic residue (I154 in the C. elegans protein) from the loop of one N-terminal domain into a pocket in the interacting N-terminal domain (van Breugel et al., 2011; Kitagawa et al., 2011c). The observed packing between the SAS-6 N-terminal head domains was used to model a nine-fold symmetric ring consistent with the central hub and spoke architecture of the cartwheel.

An important current focus of investigation is to understand how SAS-6 oligomerization is regulated to ensure that cartwheels form specifically during S-phase and only adjacent to the mother centriole. The affinity of the SAS-6 N-terminal head domains for each other is low ($K_d \sim 50-100 \mu M$ depending on the species; van Breugel et al., 2011; Kitagawa et al., 2011c), suggesting that cartwheel formation is controlled by regulatory mechanisms that promote SAS-
polymerization in the vicinity of the mother centriole. SAS-5/Ana2/STIL and the Plk4/ZYG-1 kinase, which are also required for cartwheel formation are key candidates to be involved in the regulation of SAS-6 assembly (Azimzadeh and Marshall, 2010; Nigg and Stearns, 2011; Brito et al., 2012). The kinase activities of Plk4 and ZYG-1 are required for centriole assembly (O'Connell et al., 2001; Habedanck et al., 2005) and C. elegans centriole assembly has been proposed to require phosphorylation of SAS-6 serine 123 by ZYG-1 (Kitagawa et al., 2009). Centriole assembly has also been proposed to be regulated by the phosphatase PP2A, which interacts with the SAS-5/6 complex and promotes cartwheel formation (Song et al., 2011; Kitagawa et al., 2011a).

Here, we show that SAS-6 recruitment to the mother centriole and its assembly into a cartwheel are distinct events that can be distinguished in vivo. ZYG-1 is required for SAS-6 recruitment and cartwheel assembly, but its kinase activity is only required for the latter. This finding is explained by a direct ZYG-1—SAS-6 interaction that recruits SAS-6 to the mother centriole; SAS-6 recruitment also requires a separate direct interaction between SAS-6 and SAS-5. To investigate the ZYG-1 kinase activity-dependent step following SAS-6 recruitment, we analyzed phosphoregulation of SAS-6 by ZYG-1. Surprisingly, we found that phosphorylation of the previously reported ZYG-1 target on SAS-6 (serine 123; Kitagawa, 2009) is not important for centriole assembly. The same was true for 37 additional candidate serines and threonines, identified either because they are phosphorylated by ZYG-1 in
vitro or because they are conserved among closely related nematode species. Cumulatively, the results of this analysis suggest that SAS-6 may not be the critical ZYG-1 kinase activity target for cartwheel assembly. Based on these findings, we propose that ZYG-1 binding to the SAS-6 coiled-coil recruits the SAS-6—SAS-5 complex to the mother centriole, where a ZYG-1 kinase activity-dependent step, whose target is unlikely to be SAS-6, triggers cartwheel assembly.
RESULTS

A Single-Copy Transgene Insertion System for SAS-6 in the C. elegans Embryo

To investigate the regulation of SAS-6 by ZYG-1, we used Mos1-mediated single-copy insertion (MosSCI; Frøkjaer-Jensen et al., 2008) to generate a single-copy transgene directing the expression of SAS-6::GFP. The transgene was integrated into a specific location on Chromosome II, expressed under the control of endogenous sas-6 regulatory sequences (Fig. 1A), and engineered to be resistant to RNAi-mediated depletion of endogenous SAS-6 (RR: RNAi-resistant) by altering a short region of the nucleotide sequence without affecting protein coding (Fig. 1A; Fig. S1A). Transgene-encoded wild-type SAS-6::GFP was expressed at endogenous levels (Fig. 1C), was resistant to a dsRNA targeting the altered region (Fig. 1C), and localized to centrioles (Fig. 1D).

SAS-6 depletion by RNAi prevents centriole assembly, resulting in a signature phenotype characterized by a normal first mitotic division followed by monopolar spindles during the second division (Fig. 1E; O’Connell et al., 2001). This phenotype arises because the sperm that fertilize SAS-6-depleted oocytes carry two normal centrioles, as sperm production occurs prior to introduction of the dsRNA. The sperm-derived centrioles separate and organize two spindle poles during the first embryonic division. Since no new centrioles are formed after fertilization, each daughter cell inherits only one
sperm-derived centriole leading to monopolar spindles in the second
embryonic division and 100% embryonic lethality.

The wild-type sas-6\textsuperscript{RR}::gfp transgene fully rescued both the second
division monopolar spindle phenotype and the embryonic lethality caused by
endogenous SAS-6 depletion (Fig. 1H, I). The wild-type transgene also fully
rescued the inviability of an sas-6\textsuperscript{Δ} mutant (Fig. 1I; sas-6\textsuperscript{Δ} refers to the sas-6(ok2554) allele that deletes nearly the entire sas-6 locus; see Fig. 1B). The
absence of endogenous SAS-6 was confirmed in the transgene-rescued sas-
6\textsuperscript{Δ} strain by immunoblotting (Fig. 1C). Thus, the wild-type sas-6\textsuperscript{RR}::gfp single-
copy transgene insertion is fully functional.

To validate the use of this transgenic system for the analysis of SAS-6 and
to test the role of N-terminal inter-dimer interactions in recruiting SAS-6 to
the centriole assembly site (see next section), we generated a transgene
expressing SAS-6::GFP with an I154E substitution (Fig. 1F, G). The
transgene-encoded I154E mutant SAS-6 was expressed at wild-type levels
after removal of endogenous SAS-6 (Fig. 1G). As expected from prior work
(van Breugel et al., 2011; Kitagawa et al., 2011c), the I154E mutation led to a
fully penetrant second division monopolar spindle phenotype and 100%
embryonic lethality following endogenous SAS-6 depletion (Fig. 1H, I). Thus,
the fully functional SAS-6::GFP expressed by the single copy sas-6\textsuperscript{RR}::gfp
transgene enables monitoring SAS-6 dynamics during centriole assembly and
facilitates engineering of mutations in SAS-6 to determine their phenotypic
consequences in vivo.
SAS-6 Recruitment to the Assembly Site and Cartwheel Incorporation are Distinct Events that can be Monitored In Vivo

We previously developed a method to quantitatively monitor SAS-6 levels at the site of new centriole assembly (Dammermann et al., 2008). This analysis revealed that SAS-6 is recruited to the mother centriole and reaches its maximal levels while the cartwheel is forming during S-phase. Subsequently, as cells enter mitosis, SAS-6 levels decrease by ~50% (Dammermann et al., 2008; Fig. 2D). One explanation for this dynamic behavior is that SAS-6 recruitment to the mother centriole and its incorporation into the forming cartwheel are separable events, with SAS-6 that is recruited to the mother, but not incorporated into the cartwheel, being lost following completion of cartwheel assembly (Fig. 2E). To test whether recruitment to the mother and incorporation into the cartwheel are indeed separable events, we used the SAS-6 I154E mutant which is defective for the N-terminal inter-dimer interactions underlying cartwheel formation.

To compare the recruitment profile of wild-type and I154E mutant SAS-6::GFP, we used a variation of the method described previously (Fig. 2A; Dammermann et al., 2008). In brief, the site of new centriole assembly in embryos expressing SAS-6::GFP (WT or I154E mutant) was marked by mCherry::SPD-2; SPD-2 is upstream of ZYG-1 in the centriole assembly pathway and is recruited to the mother centriole even when SAS-6 is absent (Delattre et al., 2006; Pelletier et al., 2006). The sperm centrioles in the self-
fertilizing hermaphrodites from these strains contain SAS-6::GFP, which prevents quantifying incorporation of SAS-6::GFP into the newly forming daughter centrioles. Therefore, unlabeled sperm centrioles were introduced by mating; the introduced sperm contained mCherry::histone H2B, to ensure that all imaged embryos were cross progeny. As centriolar SAS-6 does not turn over (Leidel et al., 2005; Dammermann et al., 2008) mating-derived sperm centrioles remain unlabeled after fertilization, enabling monitoring of SAS-6::GFP exclusively at the site of new centriole formation. Finally, to selectively monitor transgene-encoded SAS-6::GFP (WT or I154E mutant), endogenous SAS-6 was depleted.

Before examining the kinetics of SAS-6::GFP recruitment, we first monitored the endpoint of cartwheel formation by examining the centrosomes at the poles of the mitotic spindle. WT SAS-6::GFP formed a bright focus, representing the cartwheel of one daughter centriole, at each pole of the mitotic spindle. No signal was detected at mitotic spindle poles in embryos expressing I154E mutant SAS-6::GFP, consistent with an essential role for N-terminal inter-dimer interactions in cartwheel formation (Fig. 2B, C). As expected from prior work, formation of this SAS-6::GFP focus was not affected by depletion of SAS-4 (Fig. S2A), which is required for assembly of the outer centriole wall (Kirkham et al., 2003; Leidel and Gönczy, 2003; Pelletier et al., 2006, Dammermann, 2008).

Next, we performed a kinetic analysis of WT and I154E mutant SAS-6::GFP. This analysis was performed at 16°C because we subsequently used
the same assay to monitor SAS-6 recruitment in strains expressing temperature-sensitive ZYG-1 (see the next section). The dynamic changes in WT SAS-6::GFP at newly forming centrioles were similar to those documented previously at 21°C (Dammermann et al., 2008); SAS-6::GFP reached its maximal levels concurrent with cartwheel assembly during S-phase, and the amount of SAS-6::GFP at centrioles decreased by ~40% as cells progressed into mitosis. The I154E mutant SAS-6::GFP was also recruited to the site of new centriole assembly, reaching ~50% of the maximum observed for WT SAS-6; this localized I154E mutant SAS-6 pool subsequently declined as cells progressed into mitosis, resulting in mitotic spindle poles with no GFP focus (Fig. 2D). We conclude that SAS-6 dimers are recruited to the site of centriole assembly independently of their ability to assemble through their N-terminal domains to form the cartwheel. Thus, recruitment of SAS-6 to the mother centriole and its incorporation into the cartwheel are separable events (Fig. 2E) that can be monitored in vivo.

The Kinase Activity of ZYG-1 is Required for Cartwheel Assembly but Not for Recruitment of SAS-6 to the Mother Centriole

ZYG-1 is required to recruit SAS-6 to centrioles (Delattre et al., 2006; Pelletier et al., 2006) and its kinase activity is important for embryonic viability (O'Connell et al., 2001). However, it is not known which step in centriole assembly—SAS-6 recruitment to the mother, cartwheel assembly, or outer wall assembly—is the first to require ZYG-1 kinase activity. To determine this,
we compared the consequences of depleting ZYG-1 to those resulting from inhibiting its kinase activity by generating a zyg-1 transgenic system similar to that for sas-6 (Fig. 3A; Fig. S1B). Despite extensive efforts, we were unable to generate transgenic worms carrying the wild-type zyg-1 locus, as injection of DNA containing this locus was toxic to the worms, likely due to transient overexpression resulting in centriole overduplication. To circumvent this problem, we introduced a single temperature-sensitive point mutation (P442L; Kemphues et al., 1988; Kemp et al., 2007) into the transgene. Strains harboring transgenes with this mutation alone (control) or with an additional Kinase-Defective (KD) mutation were generated and maintained at the non-permissive temperature to keep the transgenic protein inactive (23.5°C; Fig. 3A). After injection of dsRNA targeting the endogenous locus, strains were shifted to the permissive temperature of 16°C. This approach enabled replacing endogenous ZYG-1 with transgene-encoded control- or KD-ZYG-1, with all phenotypic analysis being performed at 16°C. As a control zyg-1 transgene containing a GFP tag failed to rescue the embryonic lethality associated with endogenous ZYG-1 depletion (not shown), we generated and used untagged control and KD zyg-1 transgenes (Fig. 3A,B).

Immunoblotting and immunofluorescence confirmed that transgene-encoded control- and KD-ZYG-1 were expressed (Fig. 3B, C). ZYG-1, like its analog Plk4, is exceedingly rare and difficult to detect by immunoblotting. However, a combination of rigorous background suppression and an ultrasensitive detection method (see Experimental Procedures) allowed
detection of a band at the predicted size of ZYG-1 that was lost following RNAi-mediated depletion (Fig. 3B; the band just under ZYG-1 was of variable intensity between samples, which is indicative of a contaminant from the E. coli that worms eat). Whereas the ZYG-1 band was lost completely following zyg-1(RNAi) in the absence of a transgene, the signal declined but was not lost in the transgenic strains, as would be expected following selective depletion of endogenous but not transgene-encoded untagged ZYG-1. Thus, both transgene-encoded control- and KD-ZYG-1 appear to be expressed at near-normal levels. This conclusion was confirmed by immunofluorescence of mitotic spindles in 1-cell embryos. ZYG-1 formed a focus at each pole, and this localization was lost following zyg-1(RNAi) in the absence of a transgene. However, ZYG-1 was still detected at poles following depletion of endogenous ZYG-1 in the two transgene-containing strains (Fig. 3C). Thus, both control- and KD-ZYG-1 are expressed and localize to centrioles in vivo, indicating that the kinase activity of ZYG-1 is dispensable for its localization to the mother centriole.

We next compared the phenotypic consequences of depleting ZYG-1 to those resulting from specifically inhibiting its kinase activity. Untagged control-ZYG-1 fully rescued the embryonic lethality and monopolar second division phenotype resulting from endogenous ZYG-1 depletion (Fig. 3D). In contrast, KD-ZYG-1 failed to rescue either, indicating that ZYG-1 kinase activity is essential for centriole assembly in vivo. We next crossed the zyg-1 transgenes (integrated on Chromosome I) into the strain harboring the wild-type sas-
6RR::gfp transgene (integrated on Chromosome II) as well as expressing mCherry::SPD-2, and performed the mating-based assay as in Figure 2A (the only difference was that both endogenous ZYG-1 and SAS-6 were depleted). A clear SAS-6::GFP focus was present at mitotic spindle poles in embryos expressing control-ZYG-1, whereas embryos expressing KD-ZYG-1 lacked a detectable focus (Fig. 3E). Thus, the kinase activity of ZYG-1 is required for cartwheel assembly (Fig. 3E). Next, we determined if ZYG-1 kinase activity is required to recruit SAS-6 to the mother centriole by performing a kinetic analysis as in Figure 2D. Normal SAS-6::GFP recruitment was observed when endogenous ZYG-1 was depleted in the presence of the control-ZYG-1 transgene, and SAS-6::GFP was not recruited to centrioles when ZYG-1 was depleted in the absence of a transgene (Fig. 3F), confirming that ZYG-1 protein is essential for SAS-6 recruitment (Delattre, 2006; Pelletier, 2006). When endogenous ZYG-1 was depleted in the presence of KD-ZYG-1, a recruitment profile similar to that seen for the assembly-defective I154E SAS-6 mutant (Fig. 2D) was observed (Fig. 3F). This result indicates that ZYG-1 recruits SAS-6 to the mother centriole independently of its kinase activity; kinase activity is only required for the subsequent incorporation of SAS-6 into the cartwheel.

**ZYG-1 Directly Binds to a Specific Region of the SAS-6 Coiled-Coil that is Distinct from the SAS-5 Interaction Region**
The above experiments indicate that ZYG-1 recruits SAS-6 to the mother centriole independently of its kinase activity. To understand this function of ZYG-1, we determined if ZYG-1 directly interacted with SAS-6. We observed a robust yeast two-hybrid interaction between SAS-6 and ZYG-1 that mapped to the N-terminal region of ZYG-1 (aa 1-285, which includes its kinase domain) and the SAS-6 coiled-coil (Fig. 4A), which also interacts with SAS-5 (Leidel, 2005). To determine if this interaction is involved in the ZYG-1-dependent (but ZYG-1 kinase activity-independent) recruitment of SAS-6 to the mother centriole, we set out to identify residues in the SAS-6 coiled-coil that when mutated specifically disrupted the interaction with ZYG-1 but not SAS-5, and vice versa.

Coiled-coils are composed of two $\alpha$-helices with a heptad periodicity; positions in the heptad repeats are designated by the letters a to g. Residues in the a, d, e, and g positions typically mediate interactions between the $\alpha$-helices (Fig. 4B; Mason and Arndt, 2004). To identify the regions of the SAS-6 coiled-coil that bind to ZYG-1 and SAS-5, we therefore mutated to alanine all the charged residues in the b, c, and f positions, which have outward-facing side chains, in non-overlapping segments of 40-45 residues (Fig. 4B). Mutating 15 bcf positions in the segment containing residues 210-255 eliminated the interaction with ZYG-1 but not SAS-5; by contrast, mutation of 14 bcf residues in the 255-300 segment eliminated the interaction with SAS-5 but not ZYG-1 (Fig. 4C). Mutation of bcf residues in the 300-340 segment did not affect either interaction. All three of the bcf-to-alanine mutants still
interacted with themselves, suggesting that these mutations did not disrupt the coiled-coil. Additional mutagenesis revealed that alteration to alanine of E232, D233 and E234 (referred to as 3A\textsuperscript{ZYG-1}) or of E240 and E241 (referred to as 2A\textsuperscript{ZYG-1}) specifically disrupted the ZYG-1 interaction, whereas mutation of E286 and E287 (referred to as 2A\textsuperscript{SAS-5}) specifically disrupted the interaction with SAS-5 (Fig. 4C, S3A).

Next, we determined if the results of the two-hybrid analysis could be confirmed using binding assays with purified proteins. Purified ZYG-1\textsubscript{1-285} interacted with the WT but not the 3A\textsuperscript{ZYG-1} or 2A\textsuperscript{ZYG-1} mutant versions of the SAS-6 coiled-coil (Fig. 4D); whereas the 3A\textsuperscript{ZYG-1} or 2A\textsuperscript{ZYG-1} coiled-coil mutants retained the ability to interact with the SAS-5 C-terminus (Fig. S3B). ZYG-1\textsubscript{1-285} includes the kinase domain and a subsequent adjacent stretch of basic residues (Fig. S4A). Whereas purified ZYG-1\textsubscript{1-285} binds to the WT SAS-6 coiled-coil, binding of a truncated protein lacking the basic patch (ZYG-1\textsubscript{1-257}) is reduced to background levels (Fig. 4E), suggesting that the key SAS-6-interacting region in ZYG-1 is a basic region adjacent to the kinase domain.

Full-length WT SAS-6 bound to SAS-5 immobilized on beads at a stoichiometry of 2:1 (SAS-6:SAS-5); in contrast, no binding was detected for 2A\textsuperscript{SAS-5} mutant SAS-6 (Fig. 4F). Testing of binding to the SAS-5 N and C-termini separately indicated that the C-terminal half of SAS-5 (SAS-5\textsuperscript{CT}) binds to the SAS-6 coiled-coil, consistent with previous reports (Leidel et al., 2005; Boxem et al., 2008). Through mutagenesis and \textit{in vitro} analysis, we identified an isoleucine/arginine (IR) motif near the C-terminal end of SAS-5 that was
required for the interaction with the SAS-6 coiled-coil (Fig. 4G, S4B). This finding is consistent with a R397C mutation (Delattre et al., 2004) disrupting the SAS-5—SAS-6 interaction in a two-hybrid assay (Leidel et al., 2005). Multi-angle light scattering indicated that the SAS-5CT is a dimer (Fig. S4C), which suggests that the native SAS-6::SAS-5 complex may be a hexamer composed of two dimers of SAS-6 and one dimer of SAS-5. As we have been unable to generate full-length SAS-5 in sufficient quantities for accurate native molecular weight measurements, this is a preliminary proposal. Thus, the SAS-6 coiled-coil provides adjacent independent interaction surfaces for ZYG-1 and SAS-5 and the strategy of targeting exposed charged residues enabled identification of specific mutations that selectively disrupt these interactions.

**Direct Interactions of the SAS-6 Coiled-Coil with ZYG-1 and SAS-5 are Required to Recruit SAS-6 to the Mother Centriole**

We next tested whether the ZYG-1—SAS-6 and SAS-5—SAS-6 interactions (summarized in Fig. 5A) are important for centriole assembly in vivo by generating transgenes expressing expressing 2A\textsuperscript{ZYG-1}, 3A\textsuperscript{ZYG-1} and 2A\textsuperscript{SAS-5} mutant versions of SAS-6::GFP. All 3 mutant proteins were expressed at normal levels in the absence of endogenous SAS-6 (Fig. 5B). Following endogenous SAS-6 depletion, all three mutants resulted in 100% embryonic lethality and second division monopolar spindle formation (Fig. 5C). Crossing in mCherry::SPD-2 and measuring SAS-6::GFP at mitotic spindle poles in the mating-based assay (Fig. 2A) revealed that all three mutants failed to support
cartwheel assembly (Fig. 5D). SAS-6 is not required for ZYG-1 to localize to the mother centriole (Delattre et al., 2006; Pelletier et al., 2006) suggesting that the 2AZYG-1, 3AZYG-1 mutants do not fail in cartwheel assembly because they disrupt ZYG-1 targeting. Consistent with this, ZYG-1 localized normally following endogenous SAS-6 depletion in the 2AZYG-1 and 3AZYG-1 SAS-6 mutants (Fig. S5A).

We next tested whether the ZYG-1—SAS-6 and the SAS-5—SAS-6 direct physical interactions are required to recruit SAS-6 to the mother centriole by performing kinetic analysis of the mutant SAS-6 proteins. As shown above, depleting ZYG-1 prevents SAS-6 recruitment to the mother centriole, whereas in the presence of kinase-defective ZYG-1, SAS-6 is recruited to the mother centriole but fails to incorporate into the cartwheel (Fig. 3F). Following endogenous SAS-6 depletion the 2AZYG-1 and 3AZYG-1 mutants failed to be recruited to the mother centriole, exhibiting a phenotype analogous to ZYG-1 depletion. Thus, the direct interaction between ZYG-1 and SAS-6 is required to recruit SAS-6 to the site of new centriole assembly in vivo, explaining the kinase-activity independent role of ZYG-1 in recruiting SAS-6. The 2ASAS-5 mutant SAS-6 also failed to be recruited to the mother centriole (Fig. 5E), indicating that selectively mutating the SAS-5 interaction site on SAS-6 phenocopies SAS-5 depletion (Leidel et al., 2005).

Interestingly, imaging of sperm indicated that the 3AZYG-1, 2AZYG-1 and 2ASAS-5 SAS-6 variants are incorporated into centrioles in the presence of endogenous SAS-6, whereas I154E is not (Fig. S5B). This result suggests
that SAS-6 heterodimers containing one binding site for ZYG-1 or SAS-5 can be incorporated into the cartwheel, whereas heterodimers containing one copy of I154E SAS-6 cannot; the latter result is expected as both N-terminal heads in an SAS-6 dimer need to be interaction-competent to assemble into a cartwheel. A requirement for only a single SAS-5 interaction site per SAS-6 dimer is consistent with the 2:1 SAS-6:SAS-5CT stoichiometry measured \textit{in vitro} (Fig. 4F).

Taken together, the interaction analysis, identification and validation of specific interaction-defective SAS-6 mutations, and analysis of these mutants \textit{in vivo}, leads to the conclusion that direct interactions of ZYG-1 and SAS-5 with adjacent segments of the SAS-6 coiled-coil are essential to recruit SAS-6 to the mother centriole for cartwheel assembly.

\textbf{Phosphorylation of SAS-6 Serine 123 is Not Important for Centriole Assembly or Embryonic Viability}

While not required to recruit SAS-6 to the mother centriole, ZYG-1 kinase activity is critical for SAS-6 dimers to assemble into the cartwheel. Serine 123 of \textit{C. elegans} SAS-6 has been proposed to be a critical target of ZYG-1 kinase in centriole assembly based on comparisons of the ability of wild-type and S123A mutant \textit{sas-6} transgenes to rescue depletion of endogenous SAS-6 (Kitagawa et al., 2009). These experiments utilized random transgene insertions generated by ballistic bombardment, which are often expressed at variable levels due to germline silencing and integration at
variable copy numbers in different chromosomal sites (Seydoux and Strome, 1999; Praitis et al., 2001; Green et al., 2008). We therefore reassessed the functional importance of S123 using the single-copy targeted insertion system to generate a transgene expressing S123A mutant SAS-6::GFP (Fig. 6A). Surprisingly, we observed no significant defects in either second division spindle assembly or embryo viability with the S123A mutant (Fig. 6B), and the S123A mutant transgene fully rescued the lethality of the sas-6 deletion (Fig. 6A, B; the presence of the S123A mutation was confirmed by PCR sequencing).

The above data exclude an essential contribution of S123 phosphorylation in centriole assembly. However, it remained possible that S123 is one of multiple sites phosphorylated by ZYG-1 and that phosphorylation at S123 enhances centriole assembly. To test this, we crossed the S123A mutation into a temperature-sensitive zyg-1 mutant (zyg-1(it25); Kemphues et al., 1988; Kemp et al., 2007) and measured lethality as the temperature was increased to progressively impair ZYG-1 activity (Kemp et al., 2007). If the S123A mutation rendered centriole assembly more sensitive to reduction of ZYG-1 activity, higher lethality should be observed relative to zyg-1(it25) alone across the tested temperature range. Instead, no significant enhancement of temperature-dependent lethality was observed in the presence of S123A mutant SAS-6::GFP compared to wild-type SAS-6::GFP (Fig. 6C; for comparison, see other SAS-6 mutants in Fig. 6J that enhance zyg-1(it25) lethality). Repeating this analysis in the sas-6Δ
background yielded similar results (Fig. 6C). Thus, analysis of the S123A mutant both on its own and in the background of the zyg-1ts mutant indicates that phosphorylation of this residue by ZYG-1 does not contribute significantly to the ZYG-1 kinase-dependent control of cartwheel assembly in vivo.

**The Dominant In Vitro ZYG-1 Phosphorylation Sites In SAS-6 Contribute Only Weakly to Centriole Assembly In Vivo**

The above experiments indicate that ZYG-1 kinase activity is required for cartwheel assembly, but that the functionally relevant target is not S123 of SAS-6. We therefore analyzed phosphorylation of SAS-6 by ZYG-1 in vitro to identify other potential regulatory sites. For this purpose, we purified full-length ZYG-1, expressed in either insect cells or bacteria, and full-length SAS-6 (Fig. 6D). In vitro phosphorylation reactions followed by mass spectrometry identified a number of different target serines and threonines in the predicted unstructured C-tail, a single serine residue in a predicted break in the coiled-coil (S337), and the previously identified S123 (Fig. 6E). We next mutated S123, S337 or all 21 S/T residues in the C-tail to alanine in full-length SAS-6, or deleted the C-tail (1-389), and analyzed the effect on phosphorylation by ZYG-1 in vitro. This analysis indicated that the majority (~80%) of ZYG-1 phosphorylation in vitro is targeted to the SAS-6 C-tail (Fig. 6F, G).

To test if the newly identified phosphorylation sites are important ZYG-1 targets during cartwheel assembly in vivo, we generated Mos strains expressing alanine substitution mutants. Mutation of critical target residues in
SAS-6 is expected to phenocopy loss of ZYG-1 kinase activity and result in failure of cartwheel assembly and penetrant monopolar spindle formation. However, mutation of all 21 alanines in the C-tail, S337 on it own, or all 22 newly identified residues did not lead to penetrant monopolar spindle assembly formation in the second division (Fig. 6I). In addition the 21A and S337A mutants rescued embryonic viability both when endogenous SAS-6 was depleted by RNAi and of the sas-6Δ mutant (Fig. 6H, I). A single incidence of a monopolar spindle in the second division was observed for the 22A mutant when endogenous SAS-6 was depleted (from an n of 70), and significant embryonic lethality was also observed (Fig. 6I). Consistent with this, filming embryos through the 8-cell stage revealed a minor but appreciable increase in centriole duplication failure in the 22A mutant (Fig. 6K). As C. elegans embryos undergo over 500 individual cell divisions prior to hatching, the minor increase in centriole duplication failure likely underlies the observed embryonic lethality. Immunoblotting indicated that the different mutants were expressed at normal levels (Fig. 6H). Finally, we crossed the S337A and the 21A mutants of SAS-6 into the zyg-1(it25) temperature-sensitive mutant and measured temperature-dependent embryonic lethality. Both the S337A and the 21A enhanced the lethality of zyg-1(it25) significantly more than S123A (compare Fig. 6J to Fig. 6C), suggesting that either these mutations partially compromise SAS-6 function or that phosphorylation at these sites by ZYG-1 contributes in a non-essential manner to enhancing centriole assembly.
Overall, the analysis of ZYG-1 target sites in SAS-6, despite mutation of 22 residues mapped in vitro, failed to reveal a centriole assembly defect approaching the severity of kinase-defective ZYG-1.

**A Serine/Threonine Mutational Scan of SAS-6**

While the above effort revealed that phosphorylation of SAS-6 residues targeted by ZYG-1 in vitro is not essential for cartwheel assembly in vivo, it remained possible that the in vitro analysis failed to identify a critical residue(s). We therefore conducted a serine/threonine mutational scan of SAS-6 in vivo. Sequence alignments of SAS-6 from 5 related *Caenorhabditis* species were used to identify conserved S/T residues (Fig. S6A). We generated 8 strains, mutating sets of conserved S/T residues to alanine (Fig. 7A). 2 strains showed the expected phenotype of penetrant monopolar spindle formation in the second division and embryonic lethality and a third strain showed moderate embryonic lethality with no significant monopolar spindle formation in the second division (Fig. 7B, C), suggesting compromised SAS-6 function. Strikingly, the residues mutated in the two strains that exhibited the expected fully penetrant defect were located within the interaction surface between the N-terminal heads that is critical to form the inter-dimer interface. The set of residues in strain V (S150, T152, and S155) surround I154 in the loop that inserts into the hydrophobic pocket and the T84 residue mutated in strain III lies within the hydrophobic pocket (Fig. 7D). Based on their location, we suspected that mutation of these residues to alanine perturbed the inter-
dimer interaction (analogous to mutation of I154 to E), explaining the severe phenotype. To test if this was the case, we purified WT and mutant versions of SAS-6$^{1-389}$ (Fig. S6B). Following fractionation by gel filtration, WT SAS-6$^{1-389}$ migrated at a higher molecular weight (earlier elution) than I154E mutant SAS-6$^{1-389}$, indicative of the I154-dependent self-interaction of SAS-6 dimers. The 2 S/T>A mutants behaved identically to the I154E mutant, indicating that they are defective for head-head inter-dimer interactions (Fig. 7E), explaining their potent in vivo phenotype. The role of these S/T residues in forming the inter-dimer interface, combined with the fact that none of them are conserved outside of nematodes (van Breugel et al., 2011; Kitagawa et al., 2011c) or are detected as ZYG-1 target sites in vitro, suggests that these residues are not key regulatory sites and instead are critical for the inter-dimer interaction.

All together, analysis of 12 strains collectively mutating a total of 42 serine/threonine residues in SAS-6 failed to reveal potential phosphorylated residues whose mutation approaches the phenotypic consequences of loss of ZYG-1 kinase activity. These results suggest that SAS-6 is unlikely to be the critical ZYG-1 target during cartwheel assembly.
DISCUSSION

The experiments presented here show that centriolar cartwheel assembly occurs in two separable steps (Fig. 7F). In the first step, SAS-6 is recruited to the mother centriole in a reaction that requires direct interaction of adjacent regions on the SAS-6 coiled-coil with ZYG-1 and SAS-5. In the second step, which is controlled by the kinase activity of ZYG-1 and requires SAS-6 inter-dimer interactions, the cartwheel is formed. To tackle the question of how ZYG-1 kinase activity promotes cartwheel formation we analyzed a previously described ZYG-1 target site in SAS-6 and performed an extensive characterization of serine/threonine residues phosphorylated by ZYG-1 \textit{in vitro} and of serine/threonine residues conserved among closely related nematode SAS-6 homologs. This analysis failed to yield a residue or group of residues whose mutation approached the effect of loss of ZYG-1 kinase activity, suggesting that SAS-6 may not be the critical ZYG-1 target in the cartwheel assembly reaction. We therefore speculate, as discussed further below, that the critical target of ZYG-1 may be SAS-5, which is bound close to ZYG-1 on the SAS-6 coiled-coil and is essential for cartwheel assembly \textit{in vivo}.

\textit{A generalizable approach for the identification of specific interaction sites in coiled-coils}

Our analysis indicated that ZYG-1, but not its kinase activity, was required to recruit SAS-6 to the mother centriole. We therefore sought to explain the mechanistic basis for this requirement. Yeast two-hybrid analysis
showed that ZYG-1, like SAS-5 (Leidel et al., 2005) interacted with the SAS-6 coiled-coil. In order to identify the specific regions of the coiled-coil where these two proteins bind, we used a scanning approach, mutating all of the charged b, c and f residues in 40-45 aa regions of the SAS-6 coiled-coil to alanine. Since the two helices in a coiled-coil are held together by a combination of hydrophobic interactions between residues in the a and d positions and electrostatic interactions between residues in the e and g positions (Mason and Arndt, 2004), we expected that this method would strip a large percentage of the exposed charged residues without compromising coiled-coil assembly. This approach worked remarkably well; in addition to pinpointing short regions of the coiled-coil that harbor the ZYG-1 and SAS-5 binding sites, all of the mutants maintained the ability to interact with themselves and with partners binding outside of the mutated regions. Additional targeted mutagenesis within the identified regions then enabled identification of amino acid changes that specifically disrupted binding to ZYG-1 or SAS-5, providing a precise means to test the function of these interactions in vivo. A similar approach may prove useful in analysis of other proteins with coiled-coil regions, which are present at ~10% frequency in eukaryotic proteomes (PMID11567088) and are implicated in multiple cellular processes.

*Recruitment of SAS-6 to the assembly site requires its interaction with both ZYG-1 and SAS-5*
SAS-6 recruitment to the new centriole assembly site requires its interaction with both ZYG-1 and SAS-5. ZYG-1 targets to centrioles independently of SAS-5 or SAS-6 (Delattre et al., 2006; Pelletier et al., 2006, Fig. S5A) and interacts directly with SAS-6 (Fig. 4), explaining why the interaction with ZYG-1 is required. However, it is not clear why the interaction with SAS-5 is also required for SAS-6 to target to the site of new centriole assembly in vivo. Our biochemical data suggest that SAS-5 binds to SAS-6 in a 1:2 stoichiometry and that SAS-5 is at least a dimer (the SAS-5 C-terminus is dimeric, Fig. S4C; full length SAS-5 could potentially be a higher-order oligomer). Thus, instead of binding to both available sites in the coiled-coil of a single SAS-6 dimer, dimeric/oligomeric SAS-5 would crosslink two/multiple SAS-6 dimers. Such a hexameric (depicted in Fig. 7F) or multimeric complex may have a higher affinity for the mother centriole as it would contain multiple ZYG-1 binding sites.

As SAS-5 binds immediately adjacent to ZYG-1 on the SAS-6 coiled-coil, it is also possible that SAS-5 binding modulates the affinity of the ZYG-1—SAS-6 interaction. Alternatively, the ZYG-1 binding site on SAS-6 could be masked by another protein in vivo, and displacement of this factor by SAS-5 may be necessary for the ZYG-1 interaction. Finally, SAS-5 itself may recognize an element of the mother centriole, such as SPD-2 or ZYG-1, thereby providing a bipartite, multivalent coincidence detection mechanism for the mother centriole. However, to date, we have not observed interactions of SAS-5 with SPD-2 or ZYG-1 that would support this final possibility.
A major current limitation in testing the above ideas is the lack of full-length recombinant SAS-5 that is well-behaved in biochemical assays. Our stoichiometry measurements were performed using full-length SAS-5 concentrated on beads but we were unable to obtain sufficiently concentrated soluble full-length SAS-5 for rigorous complex analysis. Determining the molecular basis for how SAS-5 contributes to recruitment of SAS-6 to the mother centriole will be an important future goal.

*SAS-6 is unlikely to be the critical ZYG-1 target during cartwheel assembly*

The kinase activity of ZYG-1 controls cartwheel assembly, but our data suggest that SAS-6 is unlikely to be the critical ZYG-1 target in this reaction. First, we do not see any significant defect resulting from mutating the previously described S123 of SAS-6 (Kitagawa et al., 2009). We suspect the reasons for the difference between our results and the prior work are technical—the single-copy transgene insertions described here allow precise comparisons of wild-type and engineered mutants expressed at equivalent levels, which was difficult to do with ballistic bombardment, the previous method of transgenesis that resulted in random insertions of variably copy number that were susceptible to germline silencing. Although we also detected phosphorylation of S123, we find that the dominant ZYG-1 phosphorylation sites on SAS-6 *in vitro* are in the C-tail rather than in the N-terminal domain of SAS-6 as suggested in the prior work (Kitagawa et al., 2009). We believe this
difference is due to our use of full-length proteins in vitro, whereas only SAS-6 fragments were utilized as substrates in the prior study.

Despite analysis of 22 newly identified ZYG-1 phosphorylation sites in SAS-6 as well as 19 additional serine/threonine residues conserved in SAS-6 homologs from closely related nematodes, we did not find target residues in SAS-6 of similar importance to the kinase activity of ZYG-1 in cartwheel assembly. The only residues identified were located within the interaction surface between the N-terminal heads and, when mutated to alanine, disrupted inter-dimer interactions independently of phosphorylation in vitro. Of note is our simultaneous mutation of 22 residues that, while reducing ZYG-1 phosphorylation by >80% in vitro, only resulted in a very mild centriole assembly defect in vivo. While it remains formally possible that our extensive mutagenesis failed to reveal a critical residue set in SAS-6, the data suggest to us that SAS-6 is unlikely to be the key ZYG-1 target.

**SAS-5: The Critical Target of ZYG-1?**

Since ZYG-1 binds immediately adjacent to SAS-5 on the SAS-6 coiled-coil, we speculate that ZYG-1 regulates cartwheel assembly primarily by phosphorylating SAS-5, rather than SAS-6. The notion that ZYG-1/Plk4 kinases regulate cartwheel assembly by phosphorylating SAS-5/Ana2/STIL family proteins is attractive from an evolutionary perspective because, although the basic structural components of centrioles, including SAS-6 and SAS-4, are conserved across a wide range of eukaryotes, SAS-5/Ana2/STIL
and ZYG-1/Plk4 are limited to metazoans (Carvalho-Santos et al., 2010; Stevens et al., 2010; Arquint et al., 2012). This evolutionary distribution profile supports the idea that a regulatory mechanism involving phosphorylation of SAS-5/Ana2/STIL by ZYG-1/Plk4 kinases has been superimposed onto the basal centriole assembly process in metazoans. Future work will be necessary to determine if this speculation bears any merit.
EXPERIMENTAL PROCEDURES

Worm Strains and RNA-mediated Interference

*C. elegans* strains used in this study are listed in Table S1. Strains carrying integrated single-copy sas-6 and zyg-1 trangetes were constructed using the MosSCI method as previously described (Frøkjaer-Jensen et al., 2008; Fig. S1). Double-stranded RNAs were generated as described (Oegema et al., 2001) using DNA templates prepared by PCR-amplifying regions using the oligonucleotides and templates in Table S2. L4 hermaphrodites were injected with dsRNA and incubated at 16°C or 20°C depending on the experiment (for more details see extended experimental procedures).

Western Blotting

Antibodies against SAS-6 and ZYG-1 were generated by injecting GST fusions with SAS-6 aa 2-175 or ZYG-1 aa 250-371 into rabbits. Antibodies were affinity-purified from serum using standard procedures (Harlow, 1988) on columns of immobilized antigen. Worm lysates were prepared for Western blotting as previously described (Lewellyn et al., 2011). Western blots for SAS-6 were probed using 1 µg/mL of rabbit anti–SAS-6, which was detected using an HRP-conjugated secondary antibody (1:10,000; GE Healthcare Life Sciences) and a chemiluminescent detection system (ECL-Prime, GE Healthcare). Western blots for ZYG-1 were probed using 0.5 µg/mL of rabbit anti-ZYG-1, after pre-incubation with 0.2 mL of immobilized *E.coli* lysate.
(Thermo Scientific) per µg of antibody. During incubation with primary antibody, GST was also added to the antibody solution at 0.5 mg/mL. HRP-conjugated secondary antibodies were detected with the WesternBright Sirius detection system (Advansta). Blots were re-probed for α-tubulin (DM1α, Sigma) as a loading control.

**Light Microscopy and Immunofluorescence**

For details on light microscopy and immunofluorescence, see the extended experimental procedures. All quantification of fluorescence was performed with MetaMorph software (Molecular Devices) based on previous methods (Dammermann et al., 2008). Immunofluorescence of *C. elegans* embryos was performed as previously described (Oegema et al., 2001).

**Kinase Reactions and Mass Spectrometry**

ZYG-1 was purified from Sf9 cells and *E. coli* with similar results. For detailed protein purification protocols, see the extended experimental procedures. For analysis by SDS-PAGE and autoradiography, GST-ZYG-1 (0.15 µM) was incubated with GST-SAS-6 (1.6 µM), 0.2mM ATP, and 0.2 µM $^{32}$P-ATP for 20 minutes at 30°C. Reactions were terminated by the addition of sample buffer. For analysis by mass spectrometry, ZYG-1 (60 nM) was incubated with GST-SAS-6 (1.2 µM) and 0.8mM ATP for 20 minutes at 30°C (similar reactions were also performed with GST-ZYG-1). Reactions were terminated by the addition of TCA. Resuspended samples were alkylated,
trypsinized, and loaded onto a LTQ XL ion trap mass spectrometer (Thermo Scientific). Resulting MS/MS spectral data were searched using the SEQUEST algorithm against a custom made database containing human and C.elegans sequences (see extended experimental procedures for additional details).

Analytical Gel Filtration and MALS

For analysis of N-terminal domain-mediated assembly, SAS-6 variants were expressed and purified (see extended experimental procedures). SAS-6 proteins at concentrations between 20-25 µM in 20mM Tris pH 7.5, 150 mM NaCl, 1% glycerol, 1 mM DTT were injected onto a Superdex 200 gel filtration column (GE Healthcare) connected in-line to a miniDAWN TREOS 3-angle light scattering detector and an Optilab T-rEX refractive index detector (Wyatt Technology). Data were processed with ASTRA-6 software (Wyatt Technology).

For molecular weight measurements of MBP SAS-5(203-404) 6xHis, 100 µL of 15 µM protein in 25 mM HEPES pH 7.2, 400 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM DTT was injected onto a WTC-030S5 size exclusion column (Wyatt Technology) connected to the miniDAWN TREOS as above. Data analysis was performed as above.

Yeast Two-Hybrid
Two-hybrid analysis was performed according to the manufacturer’s instructions (MATCH-MAKER™, Clontech; for details see extended experimental procedures).

Pull-down assays

For all pull-down assays, prey proteins were incubated with bare resin or resin coated with bait protein. Beads were washed three times and resuspended in SDS-PAGE sample buffer before analysis on SDS-PAGE gels. For experiments to test binding between ZYG-1 and WT, 2AZYG-1, or 3AZYG-1 SAS-6 variants, final concentrations in each reaction were 12 μM SAS-6 (bait) and 4 μM ZYG-1 (prey) with 20 μL resin. For experiments to test binding between SAS-5 and WT, 2AZYG-1, or 3AZYG-1 SAS-6 variants, final concentrations in each reaction were 5 μM SAS-6 (prey) and 28 μM SAS-5 (bait) with 20 μL resin. For experiments to test binding between SAS-5 and WT or 2ASAS-5 SAS-6 variants, final concentrations in each reaction were 3 μM SAS-6 (prey) and 1 μM (for full-length) or 3 μM (for truncations) SAS-5 (bait) with 20 μL resin. See extended experimental procedures for detailed purification protocols and assay conditions.

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Chapter 2, in full, has been submitted for publication and is under review at Developmental Cell. It may appear as Molly M. Lettman, Yao Liang Wong, Valeria Viscardi, Sherry Niessen, Sheng-hong Chen, Andrew K. Shiau, Huilin Zhou, Arshad Desai, and Karen Oegema. Direct Binding of SAS-6 to ZYG-1, and Not Its Phosphorylation by ZYG-1, Recruits SAS-6 to the Mother Centriole for Cartwheel Assembly. The dissertation author was the primary researcher and author of this paper.

ABBREVIATIONS
KD=Kinase Defective; RR=RNAi-Resistant
Figure 2.1. A single-copy transgene insertion system for SAS-6 in the C. elegans embryo. (A) Schematic of the sas-6<sup>RR</sup>::gfp single copy transgene. (B) Schematic indicating the segment of sas-6 deleted in the ok2554 allele. (C) Immunoblots of worm lysates probed with antibodies to SAS-6 (top) and α-tubulin as a loading control (bottom). Lysate from worms homozygous for sas-6Δ and rescued by the sas-6<sup>RR</sup>::gfp transgene was loaded in the last lane. (D) Projected confocal image of an embryo expressing SAS-6::GFP after depletion of endogenous SAS-6. Bar, 10 µm. (E) Schematic illustrating the fate of embryos unable to duplicate centrioles due to SAS-6 depletion. (F) Schematic illustrating the effect of the I154E mutation on SAS-6 inter-dimer interactions and cartwheel assembly. (G) Immunoblot of lysates prepared from worms expressing I154E SAS-6::GFP in the absence and presence of sas-6<RNAi>. (H) Graph plotting the frequency of second division monopolar spindles observed following endogenous SAS-6 depletion in embryos from the indicted strains. (I) Graph plotting percent embryonic lethality following endogenous SAS-6 depletion in embryos derived from the indicated strains. Error bars = SD of percent lethality per worm; N = number of worms, n = total number of embryos scored.
Figure 2.2 (A-C). The assembly-defective I154E SAS-6 mutant is recruited to the mother centriole but fails subsequent cartwheel assembly. (A) Schematic of assay used to monitor SAS-6::GFP at the site of new centriole assembly. (B) Maximum intensity projection confocal images of metaphase embryos derived using the experimental scheme in (A). Sperm-derived mCherry::histone H2B weakly labels the chromosomes (arrow). Insets are magnified 3.8-fold. (C) Quantification of SAS-6::GFP fluorescence coincident with mCherry::SPD-2 for the interval 300s prior to cytokinesis, when the amount of centriolar SAS-6 is nearly constant. Measured values were normalized by dividing by the mean value for WT SAS-6::GFP. Error bars are the SEM, n=number of measurements.
Figure 2.2 (D-E). The assembly-defective I154E SAS-6 mutant is recruited to the mother centriole but fails subsequent cartwheel assembly. (D) Kinetic analysis at 16°C of SAS-6::GFP (WT or I154E) recruitment to the site of new centriole assembly using the experimental scheme shown in (A). Representative confocal projection images at indicated time points in each strain are shown. Insets are magnified 5-fold. (E) Schematic summarizing localization dynamics of WT and assembly-defective I154E mutant SAS-6. Bars, 10µm.
Figure 2.3 (A-C). The kinase activity of ZYG-1 is required for cartwheel assembly but not for recruitment of SAS-6 to the mother centriole. (A) Schematic of the zyg-1\textsuperscript{RR} single-copy transgene insertion system. (B) Schematics of transgene-encoded control and Kinase-Defective (KD) ZYG-1 and immunoblot of lysates prepared from the indicated strains and conditions probed with antibodies to ZYG-1 (top) and α-tubulin (bottom). The asterisk (*) marks a variable background band likely derived from the E. coli bacteria that worms eat, which were also used to produce the antigen for antibody generation and affinity purification. (C) Immunofluorescence analysis of ZYG-1 and SPD-2 in embryos derived from the indicated strains and conditions.
Figure 2.3 (D–F). The kinase activity of ZYG-1 is required for cartwheel assembly but not for recruitment of SAS-6 to the mother centriole. (D) Graphs plotting embryonic lethality (left) and frequency of monopolar second division (right), as in Fig. 1H & I, for the indicated strains and conditions. (E) SAS-6::GFP fluorescence at mitotic centrosomes following mating as in Fig. 2A and plotted as in Fig. 2C, in embryos expressing control, KD, or no ZYG-1 and depleted of endogenous ZYG-1 and SAS-6. (F) Kinetic analysis at 16°C of SAS-6::GFP recruitment to the site of new centriole assembly using the experimental scheme shown in Fig. 2A in embryos expressing the indicated ZYG-1 variants and depleted of both endogenous ZYG-1 and SAS-6. Representative confocal projection images of one centrosome at early and late time points in each strain are shown. Bars, 5 µm.
Figure 2.4 (A-E). Engineering specific mutations to disrupt direct interactions of adjacent regions of the SAS-6 Coiled-Coil with ZYG-1 and SAS-5. (A) Summary of yeast two-hybrid interactions between SAS-6, ZYG-1 and SAS-5. (B) Schematic of the method used to identify specific residues in SAS-6 required for ZYG-1 and/or SAS-5 binding. (C) Summary of mutagenic yeast two-hybrid analysis of the SAS-6 coiled-coil. (D-G) Analysis of the ability of control beads versus beads coated with the indicated purified proteins to bind soluble partners. Coomassie-stained gels of the binding assays are shown.
Figure 2.4 (F-G). Engineering specific mutations to disrupt direct interactions of adjacent regions of the SAS-6 Coiled-Coil with ZYG-1 and SAS-5. (D-G) Analysis of the ability of control beads versus beads coated with the indicated purified proteins to bind soluble partners. Coomassie-stained gels of the binding assays are shown. Under the conditions used, SAS-5-coated beads became saturated with SAS-6 at a SAS-6:SAS-5 stoichiometry of 2:1 calculated as a direct ratio of background-subtracted Coomassie staining intensities (F; right panel). Error bars are the SD from 4 experiments.
Figure 2.5. Direct interactions of the SAS-6 coiled-coil with ZYG-1 and SAS-5 are required to recruit SAS-6 to the mother centriole. (A) Summary of SAS-6 coiled-coil interactions and mutations engineered to disrupt each interaction in vivo. (B) Immunoblots of lysates prepared from worms expressing the indicated SAS-6 variants with or without depletion of endogenous SAS-6; blots were probed for SAS-6 (top) and α-tubulin (bottom). (C) Graphs plotting percent embryonic lethality (left) and frequency of monopolar second division (right), as in Fig. 1H & I, for the indicated conditions. (D) Graph plotting SAS-6::GFP fluorescence at mitotic centrosomes in mated embryos (see Fig. 2A-C) expressing the indicated SAS-6 variants. (E) Kinetic analysis at 16°C of recruitment of indicated SAS-6::GFP variants to the site of new centriole assembly using the experimental scheme shown in Fig. 2A.
Figure 2.6 (A-E). Neither the previously identified serine 123 nor the predominant in vitro ZYG-1 target sites in the SAS-6 C-Tail are essential for centriole assembly. (A) Immunoblot of lysates prepared from worms expressing S123A SAS-6::GFP in the absence or presence of sas-6Δ; the blot was probed for SAS-6 (top) and α-tubulin (bottom). (B) Graphs plotting frequency of monopolar second division (left) and percent embryonic lethality (right) as in Fig. 1H & I, for the indicated conditions. The WT and No Transgene data are replotted from Fig. 1. (C) Transgenes expressing WT or S123A SAS-6::GFP were introduced into a strain homozygous for the temperature-sensitive zyg-1(it25) allele. Percent embryonic lethality following endogenous SAS-6 depletion (left) or in the sas-6Δ background (right) was monitored at the indicated temperatures. (D) Coomassie-stained gels of purified tagged ZYG-1 and SAS-6. (E) Summary of phosphorylation sites on SAS-6 identified by mass spectrometry following incubation with ZYG-1 and ATP in at least 2 of 5 separate experiments. Mutations engineered to test contributions of these sites to total SAS-6 phosphorylation in vitro or to centriole assembly in vivo are marked in red. Sites in grey could not be unambiguously identified because of multiple serines/threonines within one peptide.
Figure 2.6 (F-K). Neither the previously identified serine 123 nor the predominant in vitro ZYG-1 target sites in the SAS-6 C-Tail are essential for centriole assembly. (F) Autoradiogram (top) and Coomassie-stained gel (bottom) of an in vitro kinase assay combining purified ZYG-1 with the indicated SAS-6 variants. (G) Quantification of (F); error bars represent SD of 3 reactions. (H) Immunoblots of lysates prepared from worms expressing the indicated SAS-6 variants in the absence or presence of the sas-6 deletion (Δ) or sas-6(RNAi), as indicated; blots were probed for SAS-6 (top) and α-tubulin (bottom). (I) Graphs plotting frequency of monopolar second division (left) and percent embryonic lethality (right), as in Fig. 1H & I, for the indicated conditions. (J) Transgenes expressing S337A or 21A SAS-6::GFP were introduced into a strain homozygous for the temperature-sensitive zyg-1(it25) allele. Percent embryonic lethality following endogenous SAS-6 depletion was monitored at the indicated temperatures. WT data is same as in C. (K) Centriole duplication failure rate for the indicated conditions measured over 3 rounds of division (to the 8-cell stage). n=number of divisions scored.
Figure 2.7 (A-E). A serine/threonine mutational scan of SAS-6 and a model for cartwheel assembly. (A) Serines and threonines conserved in *Caenorhabditis* species (*elegans, briggsae, brenneri, japonica, remanei; Fig. S6A*) were mutated as indicated in the schematics, to generate 8 transgenic strains. (B & C) Graphs plotting percent embryonic lethality (B) and frequency of monopolar second division (C), as in Fig. 1H & I, for the indicated conditions. (D) Schematic showing the location of serine/threonine residues in the head domain that when mutated to alanine resulted in 100% embryonic lethality and 100% monopolar cells in the second division (PDB=3PYI). (E) Analysis of SAS-6 inter-dimer interactions by gel filtration chromatography. (top) Coomassie-stained gel showing the purified WT, I154E, S/T mutant III and V variants of SAS-6<sup>1-389</sup> (Fig S6B). Elution profile of each purified protein on a Superdex 200 gel filtration column (bottom).
Figure 2.7 (F). A serine/threonine mutational scan of SAS-6 and a model for cartwheel assembly. (F) Model for cartwheel assembly in vivo. A direct interaction between ZYG-1 and the SAS-6 coiled-coil recruits the SAS-6—SAS-5 complex to the mother centriole where ZYG-1 kinase activity, whose target is unlikely to be SAS-6, promotes cartwheel assembly. SAS-5 binding is also required for recruitment to the mother but the mechanism by which this works remains to be elucidated.
Figure S2.1 (Related to Figure 1 and Figure 3). Sequence of the re-encoded regions in the sas-6 and zyg-1 RNAi-resistant transgenes. (A,B) Schematics of the sas-6::gfp (A) and zyg-1 (zg;25) (B) single-copy transgenes showing the sequence of the re-encoded regions. The DNA sequence in the re-encoded regions was altered to prevent targeting of the transgenes by dsRNAs directed against the corresponding regions of the endogenous genes, while maintaining amino acid sequence and coding bias.
**Figure S2.2** (Related to Figure 2). Cartwheel assembly is normal when outer wall assembly is prevented by SAS-4 depletion. Consistent with prior work showing that SAS-6 is recruited normally when SAS-4 is depleted (Pelletier, 2005; Dammermann, 2008), no quantitative difference in the level of SAS-6::GFP at mitotic centrosomes was detected when SAS-4 was depleted along with SAS-6 in the mating-based cartwheel assembly assay. (A) Maximum intensity projection confocal images of metaphase embryos generated using the experimental scheme in Figure 2A. The arrow indicates the sperm-derived mCherry::histone H2B. Bar, 10µm. Insets magnified 3.8-fold. (B) Quantification of SAS-6::GFP fluorescence coincident with mCherry::SPD-2 for the interval 300s prior to cytokinesis. Measured values were normalized by dividing by the mean value for WT SAS-6::GFP. Error bars are the SEM, n=number of measurements. WT data is reproduced from Figure 2B, C for comparison.
Figure S2.3 (Related to Figure 4). Mutations in SAS-6 that disrupt binding to ZYG-1 do not disrupt binding to SAS-5. (A) Extended schematic of the yeast two-hybrid assay used to identify mutations in SAS-6 that disrupt binding to ZYG-1 or SAS-5 (see Fig. 4C). (B) The 3A_{ZYG-1} and 2A_{ZYG-1} mutations in SAS-6 that disrupt ZYG-1 binding do not disrupt binding to SAS-5. Analysis of the ability of control beads versus beads coated with SAS-5^{CT} to bind WT, 3A_{ZYG-1}, or 2A_{ZYG-1} versions of the SAS-6 coiled coil. Coomassie-stained gel of the binding assay is shown.
Figure S2.4 (Related to Figure 4). SAS-5CT is dimeric. (A) A fragment of ZYG-1 encompassing the kinase domain (residues 1-285) binds to SAS-6, whereas a shorter fragment of ZYG-1 lacking an arginine-rich region outside the kinase domain (residues 1-257) does not. (B) Schematic showing the SAS-5 variants tested for binding to SAS-6. The C-terminal half of SAS-5 binds to SAS-6 and removal of the last 54 residues in SAS-5 disrupts this binding. The double (I396A, R397A) mutant disrupted SAS-6 binding, consistent with previous reports that a R397C substitution in SAS-5 disrupts SAS-6 binding (Delattre, 2004; Leidel, 2005). (C) SAS-5CT, which is sufficient for binding to SAS-6, was purified and its oligomeric state was analyzed by multi-angle light scattering (MALS). The experimentally determined molecular weight (123.8 kDa) of SAS-5CT indicates that it is dimeric (predicted dimer molecular weight 129.6 kDa).
Figure S2.5 (Related to Figure 5). SAS-6::GFP mutants that cannot bind ZYG-1 or SAS-5 can incorporate into cartwheels when endogenous SAS-6 is present. (A) The localization of endogenous ZYG-1 is not disrupted in strains expressing SAS-6::GFP transgenes that are unable to bind ZYG-1. Immunofluorescence analysis of ZYG-1 and SAS-4 in mitotic embryos derived from the indicated strains following depletion of endogenous SAS-6. Scale bar = 5 µm. (B) WT and SAS-6::GFP mutants that cannot bind ZYG-1 or SAS-5 can incorporate into sperm centrioles when endogenous SAS-6 is present, whereas SAS-6::GFP carrying the I154E mutation, which blocks N-terminal inter-dimer interactions cannot. Scale bar = 5 µm.
Figure S2.6 (Related to Figure 7). Conserved serines and threonines in SAS-6. (A) ClustalW sequence alignment of SAS-6 from different *Caenorhabditis* species. All serines and threonines are highlighted in green. Serines and threonines were selected for further analysis based on conservation in all species (red stars) or in 3/5 or 4/5 species with nearby S/T in other species (open pink stars). The selected serines/threonines were mutated in groups to alanine, as indicated by the Roman Numerals. (B) Groups of serines/threonines that abrogated centriole assembly when mutated in vivo (groups III and V) were mutated in vectors directing the expression of SAS-6<sup>1-389</sup> and the mutant proteins were purified to analyze their ability to form dimer-dimer interactions (see Fig. 7E).
<table>
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<td>unc-119(ed3) III; ltIs37 [pAA64; Ppie-1/mCHERRY::his-58; unc-119 (+)] IV</td>
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<tr>
<td>OD56</td>
<td>unc-119(ed3) III; ltIs69 [Ppie-1/mCherry-TEV-Stag::spd-2 genomic; unc-119 (+) genomic] IV</td>
</tr>
<tr>
<td>OD98</td>
<td>unc-119(ed3) III; ltSi40[pMB159/OD1227; Psas-6::SAS-6 reencoded::GFP; cb-unc-119(+)] IV</td>
</tr>
<tr>
<td>OD467</td>
<td>sas-6(ok2554) IV/nT1<a href="IV;V">qIs51</a></td>
</tr>
<tr>
<td>OD472</td>
<td>unc-119(ed3) III; ltSi40[pMB159/OD1227; Psas-6::SAS-6 reencoded::GFP; cb-unc-119(+)] IV</td>
</tr>
<tr>
<td>OD603</td>
<td>zyg-1(it25) II; ltSi40[pMB159/OD1227; Psas-6::SAS-6 reencoded::GFP; cb-unc-119(+)] IV</td>
</tr>
<tr>
<td>OD657</td>
<td>zyg-1(it25) II; ltSi40[pMB159/OD1227; Psas-6::SAS-6 reencoded::GFP; cb-unc-119(+)] IV</td>
</tr>
<tr>
<td>OD571</td>
<td>unc-119(ed3) III; ltSi79[pMB496/OD1315; Psas-6::SAS-6 (EDE232,233,234AAA) reencoded::GFP; cb-unc-119(+)] IV</td>
</tr>
<tr>
<td>OD583</td>
<td>unc-119(ed3) III; ltSi90[pMB497/OD1316; Psas-6::SAS-6 (EE240,241AA) reencoded::GFP; cb-unc-119(+)] IV</td>
</tr>
<tr>
<td>OD617</td>
<td>unc-119(ed3) III; ltSi107[pMB487/OD1228; Psas-6::SAS-6 (I154E) reencoded::GFP; cb-unc-119(+)] IV</td>
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<tr>
<td>OD471</td>
<td>unc-119(ed3) III; ltSi40[pMB159/OD1227; Psas-6::SAS-6 reencoded::GFP; cb-unc-119(+)] IV</td>
</tr>
<tr>
<td>OD594</td>
<td>unc-119(ed3) III; ltSi79[pMB496/OD1315; Psas-6::SAS-6 (EDE232,233,234AAA) reencoded::GFP; cb-unc-119(+)] IV</td>
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<td>OD748</td>
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<td>OD714</td>
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<td>OD469</td>
<td>unc-119(ed3) III; ltSi42[pMB233/OD1301; Psas-6::SAS-6(S123A) reencoded::GFP; cb-unc-119(+)] IV</td>
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<td>OD604</td>
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<td>OD478</td>
<td>sas-6(ok2554) IV; ltSi42[pMB233/OD1301; Psas-6::SAS-6(S123A) reencoded::GFP; cb-unc-119(+)] IV</td>
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</table>
Table S2.1. *C. elegans* strains, continued.

<table>
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<th>OD</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>OD659</td>
<td>zyg-1(it25) II; ltSi42[pMB233/pOD1301; Psas-6::SAS-6(123A) reencoded::GFP; cb-unc-119(+) II]; sas-6(ok2554) IV</td>
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<tr>
<td>OD506</td>
<td>unc-119(ed3) III; ltSi58[pMB258/pOD1304; Psas-6::SAS-6(337A) reencoded::GFP; cb-unc-119(+) III]</td>
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<tr>
<td>OD487</td>
<td>unc-119(ed3) III; ltSi51[pMB295/pOD1305; Psas-6::SAS-6(21A) reencoded::GFP; cb-unc-119(+) II]</td>
</tr>
<tr>
<td>OD606</td>
<td>zyg-1(it25) II; ltSi58[pMB258/pOD1304; Psas-6::SAS-6(337A) reencoded::GFP; cb-unc-119(+) II]; unc-119(ed3) III</td>
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<tr>
<td>OD605</td>
<td>zyg-1(it25) II; ltSi51[pMB295/pOD1305; Psas-6::SAS-6(21A) reencoded::GFP; cb-unc-119(+) II]; sas-6(ok2554); ltSi58[pMB295/pOD1304; Psas-6::SAS-6(21A) reencoded::GFP; cb-unc-119(+) II]</td>
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<td>OD897</td>
<td>sas-6(ok2554); ltSi51[pMB295/pOD1305; Psas-6::SAS-6(21A) reencoded::GFP; cb-unc-119(+) II]; unc-119(ed3) III</td>
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<td>OD513</td>
<td>sas-6(ok2554) IV; ltSi51[pMB295/pOD1305; Psas-6::SAS-6(21A) reencoded::GFP; cb-unc-119(+) II]</td>
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<tr>
<td>OD670</td>
<td>unc-119(ed3) III; ltSi139[pMB547/pOD1306; Psas-6::SAS-6(337A, 21A) reencoded::GFP; cb-unc-119(+) II]</td>
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<td>OD626</td>
<td>unc-119(ed3) III; ltSi113[pMB503/pOD1307; Psas-6::SAS-6(2,3,11,15,21AAAAA) reencoded::GFP; cb-unc-119(+) II]</td>
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<td>OD592</td>
<td>unc-119(ed3) III; ltSi104[pMB505/pOD1308; Psas-6::SAS-6(356A) reencoded::GFP; cb-unc-119(+) II]</td>
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<tr>
<td>OD680</td>
<td>unc-119(ed3) III; ltSi150[pMB506/pOD1309; Psas-6::SAS-6(T84A) reencoded::GFP; cb-unc-119(+) II]</td>
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<td>OD615</td>
<td>unc-119(ed3) III; ltSi105[pMB507/pOD1310; Psas-6::SAS-6(T131A) reencoded::GFP; cb-unc-119(+) II]</td>
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<tr>
<td>OD639</td>
<td>unc-119(ed3) III; ltSi126[pMB510/pOD1311; Psas-6::SAS-6(150,152,155AAA) reencoded::GFP; cb-unc-119(+) II]</td>
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<td>OD676</td>
<td>unc-119(ed3) III; ltSi146[pMB543/pOD1312; Psas-6::SAS-6(181A,182A) reencoded::GFP; cb-unc-119(+) II]</td>
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<td>OD672</td>
<td>unc-119(ed3) III; ltSi141[pMB544/pOD1313; Psas-6::SAS-6(188A,189A) reencoded::GFP; cb-unc-119(+) II]</td>
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<td>OD621</td>
<td>unc-119(ed3) III; ltSi108[pMB509/pOD1314; Psas-6::SAS-6(355,361,363,384AAAAA) reencoded::GFP; cb-unc-119(+) II]</td>
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<tr>
<td>OD762</td>
<td>unc-119(ed3) III; ltSi189[pMB564/pOD1302; Pzyg-1::ZYG-1 it25 reencoded; cb-unc-119(+) II]</td>
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<td>OD1103</td>
<td>unc-119(ed3) III; ltSi189[pMB564/pOD1302; Pzyg-1::ZYG-1 it25 reencoded; cb-unc-119(+) II]; ltSi40[pMB159/pOD1227; Psas-6::SAS-6 reencoded::GFP; cb-unc-119(+) II]; ltSi69 [Ppie-1/mCherry-TEV-Stag::spd-2 genomic; unc-119 (+) genomic] IV</td>
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<tr>
<td>OD1017</td>
<td>unc-119(ed3) III; ltSi275[pMB579/pOD1303; Pzyg-1::zyg-1K41M it25 reencoded; cb-unc-119(+) II]</td>
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<tr>
<td>OD1104</td>
<td>ltSi275[pMB579/pOD1303; Pzyg-1::zyg-1K41M it25 reencoded; cb-unc-119(+) II]; ltSi69 [Ppie-1/mCherry-TEV-Stag::spd-2 genomic; unc-119 (+) genomic] IV; ltSi40[pMB159/pOD1227; Psas-6::SAS-6 reencoded::GFP; cb-unc-119(+) II]</td>
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Table S2.2. Oligos used for dsRNA production.

<table>
<thead>
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<th>Gene</th>
<th>Oligonucleotide 1</th>
<th>Oligonucleotide 2</th>
<th>template</th>
<th>mg/mL</th>
</tr>
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<tbody>
<tr>
<td>Y45F10 D.9 (sas-6)</td>
<td>AATTAACCCCTCAGTAAAGGTATGGGAGC TAATTGAACCTCGGTAA</td>
<td>TAATACGACTCACTAGGTTATCGTTG AGCGGGTGGG</td>
<td>N2 genomic DNA</td>
<td>3.3</td>
</tr>
<tr>
<td>F59E12.2 (zyg-1)</td>
<td>AATTAACCCCTCAGTAAAGGGAGCTCTG GACGACGCAAC</td>
<td>TAATACGACTCACTAGGGAGCTCTG CCATCTCGAGATC</td>
<td>N2 genomic DNA</td>
<td>3.4</td>
</tr>
<tr>
<td>F10E9.8 (sas-4)</td>
<td>AATTAACCCCTCAGTAAAGGATGGGCTTC CGATGAAATATCG</td>
<td>TAATACGACTCACTAGGCCATGCTCTG TCAGCAACG</td>
<td>N2 genomic DNA</td>
<td>3.4</td>
</tr>
</tbody>
</table>
EXTENDED EXPERIMENTAL PROCEDURES

Worm Strains

*C. elegans* strains used in this study are listed in Table S1. To render the sas-6::gfp transgenes RNAi-resistant, the nucleotide sequence of the 294 bp at the end of the sas-6 genomic sequence was re-encoded (Fig. S1). The engineered sas-6 locus and mutant variants were cloned into pCFJ151, which contains homology arms that direct transposase-mediated insertion of intervening sequence into the ttTi5606 Mos1 insertion site on chromosome II (MosSCI; Frøkjaer-Jensen, 2008). Single-copy integrants were generated by injecting the transgene-containing plasmid (50 ng/µL) along with a plasmid encoding the Mos transposase (*Pglh-2::transposase*, pJL43.1, 50 ng/µL), and three plasmids encoding different fluorescent markers: *Pmyo-2::mCherry* (pCFJ90, 2.5 ng/µL), *Pmyo-3::mCherry* (pCFJ104, 5 ng/µL), and *Prab-3::mCherry* (pGH8, 10 ng/µL) into the strain EG4322. Injected worms were singled and moving worms lacking the fluorescent co-injection markers were identified among the progeny by visual inspection after 2-3 generations. Transgene integration was confirmed by PCR of regions spanning each side of the insertion.

To render the zyg-1 transgenes RNAi resistant, a 564bp sequence in exon 3 was re-encoded. The engineered zyg-1 locus and mutant variants were cloned into pCFJ352, which contains homology arms that direct transposase-mediated insertion of intervening sequence into the ttTi4348 Mos1 insertion
site on chromosome I. Single-copy integrants were generated by injection into the strain EG6701 and confirmed as above.

**RNA-mediated interference**

Double-stranded RNAs (dsRNAs) were prepared by using the oligonucleotides listed in Table S2 to PCR-amplify regions from N2 genomic DNA. PCR reactions were cleaned (QIAGEN) and used as templates for 50 µl T3 and T7 transcription reactions (MEGAscript, Invitrogen), which were cleaned using the MEGAclear kit (Invitrogen) and eluted into 50 µl H2O. Single-stranded RNAs (50 µl T3 and 50 µl T7) were mixed with 50 µl of 3× soaking buffer (32.7 mM Na₂HPO₄, 16.5 mM KH₂PO₄, 6.3 mM NaCl, 14.2 mM NH₄Cl) and annealed by incubating at 68°C for 10 min followed by 37°C for 30 min. L4 hermaphrodites were injected with dsRNA and incubated at 20°C for 36–46 h or at 16°C for 48-58 h before dissection and imaging of their embryos. For depletion of two targets, dsRNAs were mixed to a final concentration greater than 1 mg/mL for each RNA. For lethality assays investigating the functionality of sas-6 transgenes, worms were maintained at 20°C. L4 worms were injected with dsRNA and singled 24 hours post-injection. Adult worms were removed from the plates 48 hours post-injection and hatched larvae and unhatched embryos were counted 20 hours later. For lethality assays investigating the functionality of zyg-1 transgenes, worms were maintained at 23.5°C to keep the transgenic proteins inactive and moved to 16°C upon injection of dsRNA to deplete endogenous ZYG-1. Injected
worms were singled 48 h post injection. Adult worms were removed from the plates 72 hours post-injection and hatched larvae and unhatched embryos were counted 24-30 hours later. For lethality assays investigating the interaction between sas-6 transgenes and the zyg-1 temperature-sensitive allele, worms were maintained at 16°C. Injected worms were moved to individual plates 48 h post injection and shifted to different temperatures. Adult worms were removed 24 hours later and hatched larvae and unhatched embryos were counted 20-30 hours later, depending on the temperature.

**Western blotting**

Western blotting was performed by transferring ~80 worms into a screw-cap 1.5 mL tube containing ~0.5 mL of M9. An additional ~0.5 mL of M9 + 0.1% Triton X-100 was added and worms were pelleted by centrifuging at 400xg for 1-2 min. Worms were washed and pelleted three times in 1 mL of M9 + 0.1% Triton X-100. After the last wash, excess buffer was removed and 4× sample buffer was added to yield a final concentration of 2 worms/µL. The worms were lysed in a sonicating water bath at 70°C for 10 min, boiled in a 95°C heating block for 5 min, and sonicated again for 10 min at 70°C before freezing. Samples were thawed and loaded on SDS-PAGE. Western blots for SAS-6 were probed using 1 µg/mL of rabbit anti-SAS-6 (aa 2-175), which was detected using an HRP-conjugated secondary antibody (1:10,000; GE Healthcare Life Sciences) and a chemiluminescent detection system (ECL-Prime, GE Healthcare). Western blots for ZYG-1 were probed using 0.5 µg/mL
of rabbit anti-ZYG-1 (aa 250-371), which was pre-incubated with 0.2 mL of immobilized *E.coli* lysate (Thermo Scientific) per µg of antibody. During incubation with primary antibody, GST was also added to the antibody solution at 0.5 mg/mL. HRP-conjugated secondary antibodies were detected with the WesternBright Sirius detection system (Advansta). SAS-6 and ZYG-1 blots were subsequently probed for α-tubulin using the monoclonal DM1α antibody (1:500; Sigma-Aldrich) followed by an alkaline-phosphatase–conjugated anti–mouse secondary antibody (1:3,750; Jackson ImmunoResearch Laboratories, Inc.).

Antibodies against the N-terminus of SAS-6 were generated by using the primers in parentheses (GCGCGCGATCCACTAGCAAAATTGCATTATTGCATCA, GCGCGCGAATTCATGTGAAATTAAATGATCTCGTCG) to amplify a DNA fragment corresponding to aa 2-175 of SAS-6 from cDNA. The fragment was digested with BamHI–EcoRI and cloned into pGEX6P-1 (GE Healthcare Life Sciences). The purified GST fusion was injected into a rabbit and antibodies were purified from serum using standard procedures (Harlow, 1988) on a 1 mL NHS HiTrap column (GE Healthcare Life Sciences) containing the immobilized GST fusion after depletion of GST antibodies by sequential passage over a 5 mL NHS HiTrap column (GE Healthcare Life Sciences) containing immobilized GST. Antibodies against aa 250-371 of ZYG-1 were generated as above except the ZYG-1 antibodies were purified on a column of immobilized antigen after cleavage to remove the GST tag.
**Light Microscopy**

Images were acquired using an inverted Zeiss Axio Observer Z1 system with a Yokogawa spinning-disk confocal head (CSU-X1), a 63x 1.4 NA Plan Apochromat objective, and a QuantEM:512SC EMCCD camera (Photometrics). Acquisition parameters were controlled by AxioVision software (Zeiss).

Imaging to capture mitosis and obtain high resolution images of centrioles (panels in Fig. 2B, D) was performed by dissecting worms in M9, transferring embryos to a 2% agarose pad with a mouth pipet, and covering them with a 22x22 mm coverslip. 20 x 0.5 μm z-stacks were collected without binning; GFP (100 ms, 25% power) and mCherry (200 ms, 40% power) exposures were collected at each plane along with one central DIC section. Acquisitions were limited to ~4 z-stacks per embryo. Embryos for second division imaging to identify monopolar or bipolar cells were also mounted on agarose pads; 20 x 1 μm z-stacks were collected without binning with GFP (100 ms 30% power) exposures at each plane and one central DIC section. To monitor the recruitment of SAS-6::GFP fusions over time (Fig. 2D, 3F, 4E) embryos were mounted without compression by dissecting worms into a 5 μl drop of L-15 blastomere culture medium (Edgar, L.G., 1995) placed within a circle of vaseline on a 24 x 60 mm coverslip taped onto a metal holder. An 18x18 mm coverslip was placed on top before imaging to prevent media evaporation. 11 x 1 μm z-stacks were collected with 2 x 2 binning.
approximately every minute. GFP (100 ms, 10% laser power) and mCherry (200 ms, 50% laser power) exposures were collected at each plane along with one central DIC section. To monitor embryos to the 8-cell stage, embryos were mounted on agarose pads and 10 x 2 um z-stacks were collected without binning with GFP (100 ms 15% power) exposures at each plane and one central DIC section. To monitor SAS-6::GFP incorporation into sperm centrioles, whole worms were anesthetized in sodium azide and mounted on agarose pads. Single plane images with 100 ms GFP (20%) exposure were acquired.

For quantification of GFP signal intensity, Zeiss zvi files were partitioned into separate channels with ImageJ software. Quantification of fluorescence was performed with MetaMorph software (Molecular Devices). Z-stacks where both centrioles were fully captured were identified. A box was drawn around each centrosome in the mCherry file. A background box that was the same size as the centrosome box in one dimension and one pixel larger on each side in the other dimension was drawn around each centrosome box. The centrosome and background boxes were transferred to a maximum intensity projection of the corresponding GFP image. The per-pixel background was calculated as \([(\text{integrated intensity in background box}) - (\text{integrated intensity in centrosome box})]/[(\text{area background box}) - (\text{area of centrosome box})]. \) The GFP signal for the centrosome was the integrated intensity in the centrosome box minus the area of centrosome box multiplied by the per pixel background. The signal from both centrosomes was summed.
When the centrioles had not yet separated, both centrosomes were contained within one box and the background and centrosome boxes for the second centrosome were drawn to be the same size. Images that required a background box that went outside the embryo or went into the nucleus were discarded. To normalize to WT SAS-6::GFP over time, the WT data points for the interval when WT SAS-6::GFP levels are highest were averaged for each imaging set and all WT and mutant data points for the corresponding acquisition session were divided by this value. To assay cartwheel assembly, WT SAS-6::GFP values for the 300 seconds prior to cytokinesis were used for normalization.

**Immunofluorescence**

Worms were dissected on slides coated with subbing solution and immunofluorescence was performed as previously described (Oegema, 2001) using a 20 minute methanol fixation and rabbit-anti ZYG-1 and either rabbit-anti SPD-2 or rabbit-anti SAS-4 (1-3 µg/mL, Dammermann, 2004). Polyclonal antibodies against SPD-2, ZYG-1, and SAS-4 were directly labeled as described (Francis-Lang et al. 1999). Embryos were mounted in anti-fade solution with DAPI (Invitrogen).

**Protein purification for kinase assays**

ZYG-1 was purified from Sf9 cells and *E. coli* for comparison. Protein from both sources produced similar results. For purification of ZYG-1 from
insect cells, the zyg-1 open reading frame was PCR-amplified, adding an N-terminal Strep II tag and a C-terminal 7x Histidine tag, and cloned into the pORB transfer vector. After virus production, Sf9 cells were infected at a MOI of 10 and cells were harvested 48 hours post infection. Frozen cell pellets were lysed by light sonication after the addition of 50mM NaH$_2$PO$_4$, 300mM NaCl, 10 mM imidazole, 0.2% Tween, 1 mM $\beta$-ME, 125 mM $\beta$-glycerophosphate, pH 8, 2 mM benzamidine, EDTA-free protease inhibitor cocktail (Roche). Soluble material was added to Ni-NTA agarose (Qiagen) equilibrated in the lysis buffer, washed with the lysis buffer containing 20 mM imidazole, and eluted from the resin with the lysis buffer containing 250 mM imidazole. The eluted material was then incubated with Strep-Tactin sepharose (IBA) that had been equilibrated in 100mM Tris pH 8, 150mM NaCl, 125mM $\beta$-glycerophosphate, 1 mM $\beta$-ME, 0.2 mM benzamidine. The resin was then washed with the same buffer and bound proteins were eluted with 2.5 mM desthiobiotin in the same buffer. Proteins were snap frozen in liquid nitrogen.

For purification of ZYG-1 from E.coli, and purification of SAS-6 substrates, the open reading frames were PCR-amplified and cloned into pGEX6P-1 (GE Healthcare). Mutant versions of SAS-6 were created with the Quickchange II XL mutagenesis kit (Agilent Technologies) or provided by GenScript (21A mutant). Expression in BL21 (DE3) pLysS E. coli was induced with 0.1 mM IPTG overnight at 15°C. Cells were washed with PBS, flash frozen in liquid nitrogen, and lysed with PBS with 250 mM NaCl, 10 mM
EGTA, 10 mM EDTA, 0.1% Tween, 200 µg/mL lysozyme, 2 mM benzamidine, and EDTA-free protease inhibitor cocktail (Roche). Cells were further lysed by sonication, and soluble material was incubated with glutathione agarose equilibrated in lysis buffer. The resin was washed 5x 30 mL with PBS containing 250 mM NaCl, 1 mM β-me, and 2 mM benzamidine and bound proteins were eluted with 50 mM Tris pH 8.1, 150 mM KCl, 10 mM glutathione, and 1 mM β-me. Proteins were snap frozen in liquid nitrogen.

**Kinase Assays for Analysis by SDS-PAGE**

GST-ZYG-1 (0.15 µM) was incubated with GST-SAS-6 variants (1.6 µM) in 20mM Tris pH 7.5, 100mM KCl, 10mM MgCl2, 10mM MnCl2, 25mM β-glycerophosphate, 1mM DTT with 0.05mg/mL BSA, 0.2mM ATP, and 0.2 µM 32-P ATP for 20 minutes at 30°C. Reactions were terminated by the addition of sample buffer and analyzed by SDS-PAGE and phosphorimaging (Bio-Rad).

**Kinase Assays for Analysis by Mass Spectrometry**

StepII-ZYG-1-6XHIS (60 nM) was incubated with GST-SAS-6 (1.2 µM) in 20mM Tris pH 7.5, 100mM KCl, 10mM MgCl2, 10mM MnCl2, 25mM β-glycerophosphate, 1mM DTT with 0.8mM ATP for 20 minutes at 30°C. Reactions were terminated by the addition of TCA. Similar reactions were performed with GST-ZYG-1.
Mass Spectrometry: Liquid chromatography, Mass Spectrometry (MudPIT) Analysis, and Analysis of MS Data

Samples were resuspended in 8M Urea 50 mM Tris pH 8.0, reduced with 10 mM TCEP for 30 minutes and alkylated with 5 mM fresh IAA for 30 minutes in the dark. Samples were digested overnight in the presence of 1 mM CaCl$_2$ and trypsin at a 1:50 enzyme to substrate ratio. Digested samples were acidified to 5% final formic acid and centrifuged for 30 minutes. Peptides were loaded onto a single phase C18 column for analysis on a LTQ XL ion trap mass spectrometer (Thermo Scientific) using an reverse phase gradient (Washburn, 2001). The mass spectrometer was set in a data-dependent acquisition mode with dynamic exclusion enabled with a repeat count of 1, a repeat duration of 20s, exclusion duration 90s and an exclusion list size of 300. All tandem mass spectra were collected using normalized collision energy of 35% and an isolation window of 2 Da. One micro scan was applied for all experiments in the LTQ. Spray voltage was set to 2.50 kV. Each full MS survey scan was followed by 7 MS/MS scans.

RAW files were generated from mass spectra using XCalibur version 1.4, and MS/MS spectra data extracted using RAW Xtractor (version 1.9.1), which is publicly available (http://fields.scripps.edu/?q=content/download). MS/MS spectral data were searched using the SEQUEST algorithm against a custom made database containing human and *C. elegans* sequences. SEQUEST searches allowed for modification of STY by phosphorylation
(80.0), static modification of cysteine residues (57.0 Da due to alkylation), no enzyme specificity, and a mass tolerance set to ±1.5 Da for precursor mass and ±0.5 Da for product ion masses. The resulting MS/MS spectra matches were assembled and filtered using DTASelect2 (version 2.0.27). The validity of peptide/spectrum matches was assessed using DTASelect2 (version 2.0.27) and two SEQUEST-defined parameters, the cross-correlation score (XCorr), normalized difference in cross-correlation scores (DeltaCN). The search results were grouped by charge state (+1, +2, +3), tryptic status, and modification status (modified and unmodified peptides), resulting in 18 distinct subgroups. In each of these subgroups, the distribution of Xcorr and DeltaCN values for the direct and decoy database hits was obtained, then the direct and decoy subsets were separated by discriminant analysis. Outlier points in the two distributions were discarded. Full separation of the direct and decoy subsets is not generally possible so the discriminant score was set such that a false discovery rate of less than 1% was determined based on the number of accepted decoy database peptides (number of decoy database hits/number of filtered peptides identified×100). In addition, a minimum peptide length of seven amino acids residues was imposed and protein identification required the matching of at least two peptides per protein. Such criteria resulted in the elimination of most decoy database hits.

**Protein Purification for Analytical Gel Filtration**
SAS-6 1-389 was PCR-amplified and cloned into pGEX6P-1. The I154E mutant was PCR-amplified from an existing plasmid containing this mutation. The T84A mutant was generated using the Quickchange XL II mutagenesis kit (Agilent Technologies). The 150/152/155AAA mutant was generated using sewing PCR with mutagenic primers. The 355/361/363/384AAA mutant region was purchased from GenScript and inserted into pGEX6P-1 SAS-6 1-389.

Expression in BL21 (DE3) pLysS E. coli was induced with 0.1 mM IPTG overnight at 15°C. Cells were washed with PBS, flash frozen in liquid nitrogen, and lysed with 50 mM Tris pH 7.5, 500 mM NaCl, 1mM DTT, 200 µg/mL lysozyme, 2 mM benzamidine, 1 mM PMSF, and EDTA-free protease inhibitor cocktail (Roche). Cells were further lysed by sonication, and soluble material was obtained by centrifugation in a Ti50.2 rotor at 40,000 rpm for 30 min at 4°C. GST-tagged proteins were then purified on glutathione agarose (Sigma), eluted, and the tag was removed using Prescission Protease. Proteins were further purified using anion exchange chromatography (Mono Q, GE Healthcare) and dialyzed into 20mM Tris pH 7.5, 150 mM NaCl, 1% glycerol, 1 mM DTT.

Yeast Two-Hybrid

Two-hybrid analysis was performed according to the manufacturer’s instructions (MATCH-MAKER™, Clontech). The C.elegans sas-6 and zyg-1 open reading frames were amplified from N2 cDNA and cloned into pGBKT7 and pGADT7 plasmids. The b, c, and f positions in the C.elegans SAS-6 coiled
coil were identified using Paircoil2. SAS-6 coiled coil fragments with all b, c, and f positions mutated to alanine were purchased from GenScript. Creation of single, double, or triple mutants was performed with the Quickchange II XL mutagenesis kit (Agilent Technologies).

Protein purification for pull down assays

For experiments between ZYG-1 and WT, $3A^{ZYG-1}$, or $2A^{ZYG-1}$ versions of the SAS-6 coiled-coil, SAS-6 181-415 was PCR-amplified from the plasmids used for yeast two-hybrid and cloned into pET21a, adding a C-terminal GAG linker and 8xHistidine tag. Expression in BL21 (DE3) pLysS E. coli was induced with 0.1 mM IPTG overnight at 15°C. Cells were washed with PBS, flash frozen in liquid nitrogen, and lysed with 50 mM Tris pH 7.5, 500 mM NaCl, 1mM DTT, 200 µg/mL lysozyme, 2 mM benzamidine, 1 mM PMSF, and EDTA-free protease inhibitor cocktail (Roche). Cells were further lysed by sonication, and soluble material was obtained by centrifugation in a Ti50.2 rotor at 40,000 rpm for 30 min at 4°C. Proteins were further purified by a 35% ammonium sulfate cut. Ammonium sulfate pellets were resuspended with 15 mL of 20 mM Tris pH 8.0, 500 mM NaCl, 20 mM imidazole, 1 mM β-mercaptoethanol and centrifuged to obtain soluble resuspended material. The soluble material was then incubated with Ni-NTA resin (Qiagen) equilibrated in the same buffer for one hour. The resin was washed 2x 30 mL with 20 mM Tris pH 8.0, 500 mM NaCl, 20 mM imidazole, 1 mM β-mercaptoethanol and 2x 30 mL 20 mM Tris pH 8.0, 150 mM NaCl, 20 mM imidazole, 1 mM β-
mercaptoethanol before eluting bound proteins with 5.5 mL 20 mM Tris pH 8.0, 150 mM NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol. The eluted material was centrifuged and subjected to anion exchange chromatography on a 1 mL Mono Q column (GE Healthcare) pre-eluted with 1 M NaCl and equilibrated in 20 mM Tris pH 8.0, 200 mM NaCl, 1 mM DTT. Bound proteins were eluted with a linear gradient of increasing ionic strength in 20 mM Tris pH 8.0, 1 mM DTT. Relevant fractions were pooled and dialyzed into 20 mM Tris pH 8.0, 150 mM NaCl, 10% (w/v) sucrose, 1 mM mM β-mercaptoethanol. Fragments of ZYG-1 were PCR-amplified and cloned into pGEX6P-1. Proteins were expressed and cells were lysed as above. Soluble material was incubated with glutathione agarose equilibrated in lysis buffer. The resin was washed 5x 30 mL with 20 mM Tris pH 8.0, 500 mM NaCl, 1 mM DTT and bound proteins were eluted with 0.5 mL fractions of the same buffer containing 10 mM glutathione. Relevant fractions were pooled and dialyzed into 20 mM Tris pH 7.5, 100 mM NaCl, 10% sucrose, 1 mM β-mercaptoethanol.

For experiments analyzing binding between SAS-5 and WT, 3A ZYG⁻¹, or 2A ZYG⁻¹ versions of the SAS-6 coiled, MBP-tagged SAS-5 (203-404) 6xHis was expressed and cells were lysed as above. Soluble material was incubated with Ni-NTA agarose equilibrated in 20 mM Tris pH 8.0, 500 mM NaCl, 20 mM imidazole, 1 mM β-mercaptoethanol. The resin was washed 2x 30 mL with 20 mM Tris pH 8.0, 500 mM NaCl, 20 mM imidazole, 1 mM β-mercaptoethanol and 2x 30 mL 20 mM Tris pH 8.0, 150 mM NaCl, 20 mM imidazole, 1 mM β-mercaptoethanol before eluting bound proteins with 0.5 mL fractions of 20 mM
Tris pH 8.0, 150 mM NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol. Relevant fractions were pooled and dialyzed into 20 mM Tris pH 7.5, 100 mM NaCl, 10% sucrose (w/v), 1 mM DTT.

For experiments analyzing binding between SAS-5 and WT or 2A\textsuperscript{SAS-5} SAS-6, an E286A/E287A mutant of SAS-6 was generated by Quikchange Lightning mutagenesis (Agilent Technologies) with the following primer: 5’-TTGTGGAAAT ATGTTGAGAGCAGCACAAGGAAAAGTGGATCAGCTTC-3’. Wild-type and mutant SAS-6 were N-terminally tagged with maltose-binding protein (MBP) and plasmids were transformed into *E. coli* BL21 (DE3) pLysS cells. Protein expression was induced with 0.5 mM IPTG at 13°C overnight. Cells were pelleted and resuspended in Buffer P (25 mM HEPES/KOH pH 7.4, 300 mM NaCl, 1 mM EGTA, 0.1 mM EDTA, 10 mM β-mercaptoethanol, 10% sucrose [w/v]) supplemented with 1 mM PMSF, 1 μg/mL pepstatin A, 0.1mg/mL aprotinin, 0.1 mg/mL leupeptin and 10 μM E-64. Cell lysis was performed in a microfluidizer (Microfluidics) at 18,000 psi and the lysates were cleared by centrifugation in a Ti45 rotor (40,000 rpm, 30 min, 4°C). The resulting supernatant was incubated with 3 mL amylose resin (New England Biolabs) for 1 hr at 4°C. The resin was washed with Buffer P, and MBP SAS-6 was eluted with Buffer P + 30 mM maltose. Eluted proteins were centrifuged in a TLA120.2 rotor (100,000 rpm, 10 min, 4°C) to clear any insoluble products and flash frozen in liquid nitrogen until use. MBP SAS-5(1-202) 6xHis and MBP SAS-5(203-404) 6xHis were similarly purified. The primer used to generate the I396A/R397A mutation in the latter protein was: 5’-
CCAGCTGAACGAGAACGCCGTCGGCTGAAAAATACGCTCGCAGGAAAG
-3'. SAS-5 6xHis was expressed as above, but purification was performed over 3 mL Ni-NTA agarose. The resin was washed with Buffer P + 80 mM imidazole, and SAS-5 6xHis was eluted with Buffer P + 300 mM imidazole. The eluate was cleared and flash frozen as above.

**Pull-down assays**

For experiments to test binding between ZYG-1 and SAS-6, GST-tagged ZYG-1 fragments were dialyzed into 20 mM Tris pH 7.5, 100 mM NaCl, 20 mM imidazole, 10% (w/v) sucrose, 1 mM β-mercaptoethanol and incubated for 1 hour with either Ni-NTA agarose alone, or Ni-NTA agarose pre-bound to WT, 3A\textsuperscript{ZYG-1}, or 2A\textsuperscript{ZYG-1} SAS-6 (181-415) 8xHis. Final concentrations in each reaction were 12 μM SAS-6 and 4 μM ZYG-1 with 20 μL resin. Beads were washed three times and resuspended in SDS-PAGE sample buffer before analysis by SDS-PAGE.

For experiments to test binding between SAS-6 coiled coil fragments and SAS-5, 8xHis-tagged WT, 3A, \textsuperscript{ZYG-1} or 2A \textsuperscript{ZYG-1} SAS-6 fragments were dialyzed into 20 mM Tris pH 7.5, 100 mM NaCl, 10% (w/v) sucrose, 1 mM β-mercaptoethanol and incubated for 1 hour with either amylose resin (NEB) alone or amylose resin pre-bound to MBP SAS-5(203-404) 6xHis. Final concentrations in each reaction were 5 μM SAS-6 and 28 μM SAS-5 with 20 μL resin. Beads were washed three times and resuspended in SDS-PAGE sample buffer. Equal volumes were loaded onto SDS-PAGE gels for analysis.
For experiments to test binding between MBP-tagged SAS-6 and SAS-5 variants, proteins were subjected to Superose 12 gel filtration, concentrated in 100K MWCO Amicon Ultra concentrators (Millipore), and dialyzed into 25 mM HEPES/KOH pH 7.4, 100 mM KCl, 20 mM imidazole, 10 mM β-mercaptoethanol, 10% sucrose (w/v). MBP-tagged SAS-6 or SAS-6 2A\textsuperscript{SAS-5} were incubated for 30 minutes at 4°C with either bare Ni-NTA agarose, or Ni-NTA agarose pre-bound to SAS-5 6xHis, MBP SAS-5(1-202) 6xHis, or MBP SAS-5(203-404) 6xHis (WT or I396A/I397A). Final concentrations in each reaction were 3 μM SAS-6 and 1 μM (for full-length) or 3 μM (for truncations) SAS-5. Beads were washed three times and resuspended in SDS-PAGE sample buffer. Equal volumes were loaded onto SDS-PAGE gels for analysis.
REFERENCES


pericentriolar material proteins. Dev Cell 7, 815–829.


Chapter 3: Three Distinct Regions of SAS-6 are Required for its Recruitment to the Mother Centriole

ABSTRACT

SAS-6, which is the main structural building block of the centriolar cartwheel, is comprised of an N-terminal globular domain, a long central coiled coil, and a poorly conserved C-terminus that is predicted to be unstructured. The 9-fold symmetry of the centriolar cartwheel arises through interactions between the N-terminal globular heads of SAS-6 dimers held together by the coiled coils (Kitagawa et al., 2011c; van Breugel et al., 2011). The coiled coils distal to the heads are proposed to project outward to form the cartwheel spokes and direct assembly of the centriolar outer wall. Using the C. elegans embryo, we show that in vivo cartwheel assembly requires the unstructured C-terminal tail of SAS-6, which is not involved in N-terminal head interactions or binding to SAS-5 or ZYG-1. We show that compositional bias of the SAS-6 C-terminal tail, rather than a precise sequence, is required for centriole assembly and truncation of the SAS-6 C-terminus blocks SAS-6 recruitment to the mother centriole. We propose that the SAS-6 C-terminal tail is required for interactions in the cytoplasm with SAS-6 itself or with other binding partners, and this is required for recruitment of SAS-6 to the mother centriole and subsequent cartwheel assembly. Thus three distinct domains of SAS-6, regions that bind to ZYG-1 and SAS-5, respectively, and here, the C-terminal
tail, are required for the localization of SAS-6 to the site of new centriole assembly.
INTRODUCTION

Centrioles serve two primary functions; they template the formation of cilia and they recruit pericentriolar material (PCM) to form centrosomes. Centrioles are small cylindrical organelles whose main structural features include 1) an inner structure called the cartwheel, built from a central hub ~30 nm in diameter and nine-fold symmetric spokes emanating from the hub, and 2) an outer wall of nine-fold symmetric stabilized microtubules surrounding the cartwheel (Loncarek and Khodjakov, 2009; Azimzadeh and Marshall, 2010; Carvalho-Santos et al., 2011; Nigg and Stearns, 2011; Brito et al., 2012; Gönczy, 2012).

Over the last decade, many of the genes required for centriole assembly have been identified. SAS-6 and SAS-4 are present in all organisms that build centrioles and are thought to have evolutionarily conserved structural roles in the cartwheel and outer wall, respectively (Carvalho-Santos et al., 2010; Hodges et al., 2010). In metazoans, centriole assembly also requires the STIL/Ana2/SAS-5 family of proteins (Delattre et al., 2004; Stevens et al., 2010; Castiel et al., 2011; Kitagawa et al., 2011b; Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012) and is controlled by the kinase PLK4/ZYG-1 (O’Connell et al., 2001; Bettencourt-Dias et al., 2005; Habedanck et al., 2005). In C.elegans, SPD-2 is required for ZYG-1 localization (Delattre et al., 2006; Pelletier et al., 2006), a role that is filled by Asterless in Drosophila (Dzhindzhev et al., 2010) and may be partially performed by
Cep152 in human cells (Cizmecioglu et al., 2010; Hatch et al., 2010; Slevin et al., 2012).

Because of RNAi depletion studies coupled with time resolved electron microscopy in the nematode *C. elegans*, the organism where many of the genes required for centriole assembly were originally identified (O’Connell et al., 2001; Kirkham et al., 2003; Leidel and Gonczy, 2003; Dammermann et al., 2004; Delattre et al., 2004; Kemp et al., 2004; Pelletier et al., 2004; Leidel et al., 2005), we can separate centriole assembly into two temporally and genetically distinct steps (Pelletier et al., 2006). The first step, cartwheel assembly, requires SPD-2, the kinase ZYG-1, SAS-5, and SAS-6. The second step, assembly of the outer microtubule wall, requires SAS-4 and α-, β-, and γ-tubulin (Pelletier et al., 2006).

Recent work has implicated SAS-6 as the main structural component of the centriolar cartwheel. SAS-6 proteins throughout evolution are comprised of an N-terminal globular domain, a long central coiled coil, and a C-terminal tail that is poorly conserved and not predicted to be structured. Structural studies on the SAS-6 N-terminus from different organisms pointed to an evolutionarily conserved model where SAS-6 forms parallel dimers via its coiled coil domain and subsequent interactions between the globular N-terminal heads of SAS-6 dimers drive assembly of the cartwheel (Kitagawa et al., 2011c; van Breugel et al., 2011). In this model, the N-terminal globular domains of SAS-6 build the central hub of the cartwheel and the coiled coils make up the spokes. This leaves the SAS-6 C-terminal tails pointing outward toward the microtubule
wall, which is consistent with immuno-gold labeling of a C-terminally tagged SAS-6 in *Chlamydomonas reinhardtii* (van Breugel et al., 2011).

In this study, we aimed to understand the importance of the unstructured C-terminal tail of SAS-6. Using a robust gene replacement system, we found that the C-terminal tail of SAS-6 is required for assembly of the centriolar cartwheel, but a precise sequence within the C-tail is not. We further show that the C-tail is required for recruitment of SAS-6 to the site of new centriole assembly, and propose models underlying this requirement.
RESULTS

The SAS-6 C-terminal tail is required for centriole assembly

SAS-6 family proteins have a C-terminal tail (C-Tail) following the coiled coil domain that is predicted to be unstructured. The C-Tail, which is 86 amino acids long in C. elegans, is poorly conserved in primary sequence and length (Fig. 1A). To test if the C-Tail is important for centriole assembly, we generated single copy integrated transgenes encoding GFP fused to the C-terminus of a series of C-terminal SAS-6 truncations (Lettman et al.; Frøkjaer-Jensen et al., 2008; Fig. 1B). These truncated GFP fusions were expressed at levels comparable to wild type (WT) SAS-6::GFP in vivo (Fig. 1C).

Failure in centriole assembly in C.elegans results in an easily identifiable monopolar second division phenotype. This is explained by the fact that sperm centrioles, which are brought into the acentriolar oocyte, separate and recruit PCM to form a normal bipolar first division, but each daughter cell inherits only a single centriole, resulting in monopolar spindles in the second embryonic division (O’Connell et al., 2001; Fig. 1E). Sperm centrioles are built prior to the time at which RNAi depletions are performed, and are thus unaffected. SAS-6::GFP fusions with short truncations of the SAS-6 C-terminus complemented the loss of endogenous SAS-6 by RNAi depletion or deletion of the sas-6 genomic locus ((sas-6(ok2554); Fig. 1C, D, F); however, SAS-6 1-424, 1-415, and 1-388, which eliminated between 68 and 104 residues from the C-terminus, caused embryonic lethality (Fig. 1D) and led to increasingly penetrant second division monopolar spindles (Fig. 1F). Failure to
The complement was not due to instability of the GFP-tagged protein in the absence of endogenous SAS-6 (Fig. 1G). Thus, the SAS-6 C-Tail is necessary for centriole assembly in vivo.

**Compositional bias of the SAS-6 C-terminal tail, rather than a specific sequence, is required for centriole assembly**

We next wanted to further examine the SAS-6 C-terminal tail and determine if a precise sequence is required for centriole assembly. Because truncation of the C-terminal tail of SAS-6 at amino acid 454 does not cause a centriole assembly defect (Fig. 1D, F) and can fully rescue a strain homozygous for a deletion of the sas-6 genomic locus (Fig. 1C), while truncation at amino acid 424 leads to fully penetrant embryonic lethality (Fig. 1D), we first made an internal deletion of residues 425-454 to determine if deletion of this region results in the same phenotype as truncation at residue 424, which would suggest the important sequence element lies within this region (mutant I, Fig. 2A). While depletion of endogenous SAS-6 in the strain expressing SAS-6(425-454Δ)::GFP had a modest effect in the second division monopolar cell assay and resulted in significant embryonic lethality (Fig. 2B, C), it was not as severe as truncating SAS-6 at amino acid 424 (compare to Fig. 1D, F), suggesting there are sequence features of SAS-6 just N-terminal to amino acid 424 that are compromised by the absence of any sequence (truncation at 424), but that can partially function when additional residues (amino acids 455-492) are included.
The dominant features of the SAS-6 C-terminal tail throughout evolution are an enrichment in the amino acids Serine, Threonine, and Proline (Table S1), and a lack of predicted secondary structure. We had previously shown that replacement of all 21 Serines and Threonines with Alanine in the C-terminal tail results in a very mild in vivo phenotype that can only be detected in the background of a mutant with compromised centriole function (Lettman et al.), so we focused our efforts on understanding the role of Prolines or other compositional bias of the SAS-6 C-terminal tail. To examine what features of the SAS-6 C-terminal tail are important, we made a series of replacements of the tail in vivo (Figs. 2A, S1). We shuffled the amino acids within the SAS-6 tail (mutants II, III, and IV), mutated the Prolines to Alanine (mutants V and VI), or replaced the tail with non-SAS-6 sequence based on the average composition of C.elegans centrosomal proteins, modified to ensure that no predicted secondary structure was created (mutants VII and VIII). Only replacing the entire C-terminal tail (amino acids 409-492) with a randomly generated, non-SAS-6 sequence (mutant VIII) approached the level of phenotypic severity in both the second division and embryonic lethality assays that results from truncating the SAS-6 C-terminal tail (Fig. 2B, C). Although the randomly generated tail sequence is not predicted to make an ordered structure, we cannot exclude the possibility that this sequence of amino acids has a dominant effect that explains its severe phenotype. Nevertheless, we can conclude that no precise sequence, but rather a compositional bias, in the SAS-6 C-terminal tail is required (mutants IV and VI, Fig. 2B, C).
Deletion of the SAS-6 C-terminal tail does not affect the interaction of SAS-6 with ZYG-1 or SAS-5 or between the SAS-6 N-termini

We wanted to understand why centriole assembly fails in the absence of the SAS-6 C-terminal tail, so we next investigated whether the C-terminal tail is important for interaction with SAS-6 binding partners. Several SAS-6 interactions are known to be required for centriole assembly: 1 & 2) recognition of the SAS-6 coiled coil by ZYG-1 and SAS-5 and 3) interactions between the SAS-6 N-terminal domains (Lettman et al.; Leidel et al., 2005; Kitagawa et al., 2011c; van Breugel et al., 2011; Qiao et al., 2012). Previous studies have mapped the residues in SAS-6 that are important for binding to ZYG-1 and SAS-5 (Lettman et al.; Qiao et al., 2012), and these residues fall within the SAS-6 coiled coil. We therefore would not expect that the SAS-6 C-terminal tail would be required for these interactions, but it is possible that the SAS-6 C-terminal tail modulates these interactions (Fig. S2A). Yeast two-hybrid experiments showed no difference in binding to ZYG-1 between full length SAS-6 and the SAS-6 coiled coil alone (residues 181-415; Fig. S2B). Pull down experiments (Fig. S2C) revealed that full length and C-terminally truncated SAS-6 (residues 1-389) bind SAS-5 203-404 attached to nickel agarose beads with similar efficiency. Thus, deletion of the C-Tail does not affect centriole assembly by perturbing the binding of SAS-6 to ZYG-1 or SAS-5.
We next determined whether the C-Tail controls interactions between the SAS-6 N-terminal domains. Prior work had shown that *C. elegans* SAS-6 lacking its C-Tail interacts through its N-terminal domains to form higher order oligomers *in vitro*, and that this interaction is abrogated by the I154E mutation (Kitagawa et al., 2011c). Consistent with these results, we observed a clear shift to larger hydrodynamic radius (reduced retention volume) for SAS-6 1-389 compared to SAS-6 1-389 I154E (50 µM loaded for SAS-6 1-389 vs. 60 µM for SAS-6 1-389 I154E; Fig. S2D, E). The I154E mutation similarly disrupted the shift to larger hydrodynamic radius by the full length SAS-6 molecule (Fig. 2E; due to the lower solubility of wild type SAS-6, full length SAS-6 was loaded at 25 µM and full length SAS-6 I154E was loaded at 55 µM). Thus, deletion of the SAS-6 C-Tail does not significantly inhibit interactions between the SAS-6 N-terminal domains or binding to ZYG-1 or SAS-5 *in vitro*, suggesting that the tail provides a mechanistically distinct contribution during centriole assembly *in vivo*.

**The SAS-6 C-terminal tail is required for cartwheel assembly in vivo**

We next sought to understand which step of centriole assembly is affected by truncation of the SAS-6 C-terminal tail. Centriole assembly in the *C. elegans* embryo can be separated into two distinct steps where first the cartwheel is built, then the outer microtubule wall (Pelletier et al., 2006; Fig. 3A). Cartwheel assembly occurs during S-phase and requires SAS-6, SAS-5, the kinase ZYG-1 and the scaffold protein SPD-2 (O'Connell et al., 2001;
Construction of the outer microtubule wall occurs during mitotic prophase and requires SAS-4, γ-tubulin and α/β-tubulin (Pelletier et al., 2006; Dammermann et al., 2008); this step must also involve interactions between outer wall proteins and cartwheel components. In particular, the geometry of SAS-6 oligomers, with the coiled coils projecting out from the central hub, suggests potential involvement of the coiled coil and the C-Tail in the second step of outer wall assembly.

To define the step in centriole assembly compromised by loss of the SAS-6 C-terminal tail, we monitored new centriole assembly in embryos expressing either WT or 1-424 SAS-6::GFP. When cartwheel assembly is successful, but outer wall formation is blocked, SAS-6 localizes normally (Pelletier et al., 2006; Dammermann et al., 2008). When cartwheel assembly fails, however, no SAS-6 localization can be detected at first division mitotic centrosomes (Lettman et al.).

To monitor the two distinct assembly steps in vivo, we used an established assay (Lettman et al.; Dammermann et al., 2008). We marked the site of new centriole assembly with mCherry::SPD-2; SPD-2 is upstream of SAS-6 in centriole assembly (Delattre et al., 2006; Pelletier et al., 2006) and is recruited even when SAS-6 is absent. To prevent contribution from SAS-6::GFP that is stably incorporated into sperm centrioles (Leidel et al., 2005; Dammermann et al., 2008), we mated the hermaphroditic SAS-6::GFP, mCherry::SPD-2 expressing mothers to males with unlabeled centrioles.
These males also expressed mCherry::histone so that we could confirm that the embryos we monitored were the product of mating. In addition, endogenous SAS-6 was depleted from the oocytes in all of the experiments (Fig. 3B).

Using this experimental scheme, we found that, as expected, WT SAS-6 formed a GFP focus at each pole of the mitotic spindle during the first embryonic division. We next tested if SAS-6 1-424, which is the smallest C-Tail truncation that fails to support centriole formation, had defects in cartwheel assembly. Since the SAS-6 C-Tail is thought to be near the outer edge of the cartwheel (van Breugel et al., 2011), we expected that SAS-6 1-424 would not affect the first step of cartwheel assembly and localize normally, but instead fail in the second step of outer wall assembly. In contrast to this expectation, embryos expressing 1-424 SAS-6::GFP that went on to fail centriole assembly did not give rise to any GFP signal at mitotic spindle poles (Fig. S2A, B), indicating that the SAS-6 C-tail is required for cartwheel formation in vivo.

*The SAS-6 C-terminal tail is required for recruitment to the site of new centriole assembly*

We previously showed that cartwheel assembly can be further dissected by monitoring SAS-6::GFP localization over time at 16°C (Lettman et al.). At 16°C, rather than our typical ~21°C assay conditions, the cell cycle slows down considerably and the short window of SAS-6 recruitment distinct
from assembly can be visualized. In this assay, mutants that disrupt cartwheel assembly but do not affect SAS-6 localization, such as the SAS-6 I154E mutation that blocks assembly of SAS-6 dimers through their N-termini (Kitagawa et al., 2011c), are recruited to ~40% of WT levels and subsequently lost during mitosis when cartwheel assembly has failed (Lettman et al.; Fig. 3D). To determine if the SAS-6 C-terminal tail is required for SAS-6 localization, rather than cartwheel assembly per se, we monitored the recruitment of WT, 1-424, or 1-388 SAS-6::GFP over time at 16°C using the experimental scheme shown in Fig. 3B. Surprisingly, when we extended the cell cycle by keeping embryos at 16°C, in embryos expressing 1-424 SAS-6::GFP, rather than a centriole assembly failure rate of 47%, centriole assembly failed only 9% of the time (Figs. 1F, 3C), suggesting that loss of the SAS-6 C-terminal tail does indeed disrupt SAS-6 localization and that the partially functional SAS-6 1-424, when given more time, can successfully localize and build a cartwheel. In line with this result 1-388 SAS-6::GFP, which never succeeds in centriole assembly (Figs. 1F, 3C), fails to be recruited to the site of centriole assembly at any timepoint (Fig. 3D).
DISCUSSION

The experiments presented here show that the SAS-6 C-terminal tail is essential for centriole assembly. Increasingly long truncations of the SAS-6 C-terminal tail lead to increasingly penetrant defects in centriole assembly (Fig. 1F). The C-terminal tail of SAS-6 is required for centriole assembly because SAS-6 cannot be recruited to the mother centriole without it (Fig. 3D). Complete shuffling of the sequence of the SAS-6 C-terminal tail (residues 409-492), however, results in no detectable defect in centriole assembly in early divisions and only a minor increase in embryonic lethality (mutant IV; Fig. 2B, C). Possible mechanisms explaining why the SAS-6 C-terminus, but no precise sequence therein, is required for localization of SAS-6 to the site of new centriole assembly are discussed further below.

The SAS-6 C-terminal tail is required, but no precise sequence is required

In the last ten year, an arena of protein science that has emerged is the study of intrinsically disordered proteins. Mounting evidence suggests that regions of intrinsic disorder within proteins are required for function in vivo (see Uversky and Dunker, 2010; Dyson, 2011; Uversky, 2011; Tompa, 2012 for recent reviews). This intrinsic disorder is postulated to be required for a variety of functions including post-translational modification, efficient degradation, linking multiple domains, the ability to bind different partners, the ability to find interaction partners because of a large radius (fly-casting),
having a large interaction surface to allow for high specificity/low affinity interactions, the ability to adopt multiple conformations. Although not formally shown to be disordered, the SAS-6 C-terminal tail lacks predicted secondary structure. Additionally, it is not well conserved in either amino acid sequence or length, yet the overall composition is relatively constant and consistent with the composition of intrinsically disordered proteins (Uversky and Dunker, 2010; Brown et al., 2011; Uversky, 2011; Mosca et al., 2012). The C-terminal tail of SAS-6 could be involved in any of the above processes. We do not favor the idea that the C-tail is a target of Serine/Threonine phosphorylation, because a mutant in which all the S/T in the C-tail have been changed to Alanine rescues a genetic deletion of SAS-6 (Lettman et al.). Additionally, although a KEN box is present in the C-terminus of SAS-6 in several species and has been shown to be required for degradation of human SAS-6 (Strnad et al., 2007), we suspect that is a pathway distinct from the results described here because we do not see excessive centriole assembly or formation of aggregates in the cytoplasm when the SAS-6 C-terminal tail has been truncated. The other postulated functions for disordered regions involve protein-protein interactions. Although no known SAS-6 interaction has been mapped to the SAS-6 C-terminus, the possibility remains that the function of this region is indeed to act as an interaction interface, and this possibility is discussed further below.
The SAS-6 C-terminal tail, as well as interaction with SAS-5 and ZYG-1, is required for localization

We previously showed that SAS-6 interaction with both SAS-5 and ZYG-1 is required for SAS-6 targeting to the mother centriole (Lettman et al.). Here we show that the SAS-6 C-terminal tail is also required for localization, but not because it is required for the above interactions. It is yet unclear why three distinct regions of SAS-6 would be required to bring SAS-6 to the mother centriole. As described above, an idea to explain why the SAS-6 C-terminal tail is required for localization, and thus centriole assembly, is that it is a binding site for an interaction partner. This would mean three distinct associations with SAS-6 are required for localization. It is possible that multiple associations are necessary to prevent the localization of too much SAS-6 and therefore prevent the formation of too many centrioles. A long-standing mystery in centriole assembly is how only a single daughter centriole is built per mother centriole. One way of restraining overduplication could be limiting the local pool of centriole components. A related idea is that each interaction is sequential and prepares SAS-6 for cartwheel assembly.

C-tail: binding site for unidentified SAS-6 interaction partner?

As described above, we postulate that the SAS-6 C-terminal tail is required for interaction with a binding partner. This binding partner could be another protein or SAS-6 itself. A recent study suggested that full length SAS-6 is able to form an anti-parallel tetramer (Qiao et al., 2012). Formation of the
tetramer was postulated to be mediated by a long interaction interface comprised of regions of alternating positive and negative charges along the C-terminal half of the coiled coil and the C-terminal tail. This long interaction interface is consistent with interactions mediated by regions of disorder (Gunasekaran et al., 2003) and regions of alternating charge mediating this interaction is consistent with both the lack of sequence conservation of SAS-6 and the lack of requirement of a precise sequence. It was shown that binding of SAS-5 to the SAS-6 coiled coil prevented formation of the tetramer, although the affinity of the SAS-6 dimer-dimer interaction was very low and thus we could see no increase in SAS-5 binding to SAS-6 lacking the C-terminal tail in our assays (Qiao et al., 2012; Fig. S2C). If the anti-parallel tetramer does indeed form in vivo and the C-terminal tail is required for this conformation, three possible functions of this conformation are 1) to prevent premature binding to SAS-5, 2) as an auto-inhibited conformation to prevent aberrant centriole assembly, or 3) to create a binding site for a different protein. We exclude the first and second possibilities because if SAS-6 was prematurely binding to SAS-5, we would expect to see an increase in SAS-6 localization, not a total loss of localization and if the anti-parallel tetramer is an auto-inhibited conformation, we would expect to see aberrant assemblies of SAS-6 when this conformation is blocked by removal of the C-tail. Neither of those models is consistent with our results. Thus we favor the idea that if the function of the C-tail is for mediating anti-parallel tetramer formation, this conformation is a binding site for a different protein.
As yet no SAS-6 interaction has been mapped to the SAS-6 C-terminus. Two known, unmapped SAS-6 interaction partners are SUR-6 and PAA-1, subunits of the PP2A complex (Kitagawa et al., 2011a). We wondered if the SAS-6 C-tail was mediating this interaction, but in our experiments, we could not confirm the interaction of full length or C-terminally truncated SAS-6 with PP2A components (data not shown).

Thus we speculate that the SAS-6 C-tail is required for interaction with an as yet unidentified binding partner, possibly through the formation of an anti-parallel tetramer conformation (Fig. 4).
EXPERIMENTAL PROCEDURES

Worm Strains

*C. elegans* strains used in this study are listed in Table S2. Strains carrying single copy integrated sas-6 transgenes were generated as described (Lettman et al.; Frøkjaer-Jensen et al., 2008).

RNA-mediated interference

Double-stranded RNAs (dsRNAs) were prepared using the oligonucleotides in Table S3 as described (Lettman et al.). L4 hermaphrodites were injected with dsRNA and incubated at 20°C for 36–46 h (Figs. 1, 2, S3) or 16°C for 48–58 h (Fig. 3) before dissection and imaging of their embryos. For lethality assays, L4 worms were injected with dsRNA and moved to individual plates 24 hours post-injection. Injected worms were removed from the plates 48 hours post-injection and hatched larvae and unhatched embryos were counted 20 hours later.

Western blotting

Sample preparation and western blotting were performed as described (Lettman et al.). Briefly, individual worms were moved into a tube, washed, and lysed by sonication and boiling. Samples were prepared to a final concentration of 2 worms per µL and 20 worms were loaded per lane. After SDS-PAGE and transfer to nitrocellulose, blots were probed with antibodies to
the N-terminus of SAS-6 (residues 2-175) and detected using an HRP-conjugated secondary antibody (GE Healthcare). The same blots were reprobed for α-tubulin (DM1α, Sigma-Aldrich) followed by an alkaline-phosphatase–conjugated anti–mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc.).

**Yeast Two-Hybrid**

Two-hybrid analysis was performed according to the manufacturer’s instructions (MATCH-MAKER™, Clontech). The sas-6 and zyg-1 open reading frames or indicated fragments were PCR-amplified and cloned into pGBK7 and pGADT7 plasmids. Creation of the ZYG-1 K41M mutant was performed with the Quickchange II XL mutagenesis kit (Agilent Technologies).

**Light Microscopy**

Images were acquired with an inverted Zeiss Axio Observer Z1 system with a Yokogawa spinning-disk confocal head (CSU-X1), a 63x 1.4 NA Plan Apochromat objective, and a QuantEM:512SC EMCCD camera (Photometrics). Acquisition parameters were controlled by AxioVision software (Zeiss). To capture images of mitosis (**Fig. S3**), worms were dissected in M9 and embryos were transferred to a 2% agarose pad and covered with a 22 x 22 mm coverslip. 20 x 0.5 μm z-stacks (alternating GFP (100 ms, 25% power) and mCherry (200 ms, 40% power) and one central DIC) were collected without binning. Acquisitions were limited to ~4 z-stacks per embryo. To
monitor the second embryonic division (Figs. 1F, 2B), embryos were also mounted on agarose pads; 20 x 1 µm GFP z-stacks (100 ms 30% power) and one central DIC section were collected without binning. To monitor the recruitment of SAS-6::GFP fusions over time (Fig. 3C, D), the microscope room was chilled to and maintained at 16°C. Worms were dissected into a 5 µl drop of L-15 blastomere culture medium (Edgar, L.G., 1995) on 24 x 60 mm coverslip taped onto a metal holder. To prevent compression, a circle of Vaseline surrounded the worm drop and an 18 x 18 coverslip was placed on top to prevent evaporation. Alternating GFP (100 ms, 10% laser power) and mCherry (200 ms, 40% laser power) 11 x 1 µm exposures were collected along with one central DIC section with 2 x 2 binning. Acquisitions were limited to ~20 z-stacks per embryo. Quantification of GFP signal intensity (Figs. 3D, S3B) was performed as described (Lettman, 2012).

Protein Purification for Analytical Gel Filtration

For purification of untagged full length wild type SAS-6, untagged full length SAS-6 I154E, and untagged wild type SAS-6 1-389, the region of interest was PCR-amplified and cloned into pRSETa, removing the histidine tag in pRSETa. I154E mutants were generated from these vectors using the Quickchange XL II mutagenesis kit (Agilent Technologies). Expression in BL21 (DE3) pLysS E. coli was induced with 0.1 mM IPTG overnight at 15°C. Cells were washed with PBS, flash frozen in liquid nitrogen, and lysed with 50 mM Tris pH 7.5, 500 mM NaCl, 1mM DTT, 200 µg/mL lysozyme, 2 mM
benzamidine, 1 mM PMSF, and EDTA-free protease inhibitor cocktail (Roche). Cells were further lysed by sonication, and soluble material was obtained by centrifugation in a Ti50.2 rotor at 40,000 rpm for 30 min at 4°C. Proteins were further purified by ammonium sulfate cut (20% for full length SAS-6, 35% 1-389). Ammonium sulfate pellets were resuspended with 10 mL of 25 mM Tris pH 7.5, 250 mM NaCl, 1 mM DTT, 0.2 mM benzamidine, 0.1 mM PMSF and centrifuged to obtain soluble resuspended material. The soluble material was then subjected to a reversible low salt precipitation by dilution with 20 mM sodium phosphate pH 6.0, 1 mM DTT, 0.2 mM benzamidine, 0.1 mM PMSF (to ~60 mM NaCl for full length SAS-6 and ~30 mM NaCl for SAS-6 aa 1-389). The precipitated material was centrifuged in a Ti45 rotor at 40,000 rpm for 35 min. The supernatant was removed and the pellet was resuspended gently into a minimal volume of 50 mM Tris pH 8.0, 500 mM NaCl, 1 mM DTT and centrifuged (~4 mL final). The supernatants were diluted to ~200 mM NaCl with 20 mM Tris pH 8.0, 1 mM DTT, centrifuged and subjected to anion exchange chromatography on a 1 mL Mono Q column (GE Healthcare) pre-eluted with 1 M NaCl and equilibrated in 20 mM Tris pH 8.0, 1 mM DTT and 200 mM NaCl (full length) or 180 mM NaCl (1-389). Bound proteins were eluted with a linear gradient of increasing ionic strength in 20 mM Tris pH 8.0, 1 mM DTT. SAS-6 1-389 I154E was expressed and purified as above, using a 35-45% ammonium sulfate cut and omitting the low salt precipitation step.

**Analytical Gel filtration**
For analysis of N-terminal domain mediated assembly, SAS-6 proteins at concentrations between 25-60 µM in 20mM Tris pH 7.5, 150 mM NaCl, 1% glycerol, 1 mM DTT were injected onto a WTC 030S5 gel filtration column connected in-line to a miniDAWN TREOS 3-angle light scattering detector and an Optilab T-rEX refractive index detector (Wyatt Technology). Data were processed with ASTRA-6 software (Wyatt Technology).

**Protein purification for in vitro pull-down assays**

Full length and 1-389 SAS-6 were N-terminally tagged with maltose-binding protein (MBP) and proteins were expressed and purified in *E. coli*. Cells were pelleted and resuspended in Buffer P (25 mM HEPES/KOH pH 7.4, 300 mM NaCl, 1 mM EGTA, 0.1 mM EDTA, 10 mM β-mercaptoethanol, 10% sucrose [w/v]) supplemented with 1 mM PMSF, 1 µg/mL pepstatin A, 0.1mg/mL aprotinin, 0.1 mg/mL leupeptin and 10 µM E-64. Cells were lysed and soluble material was incubated with amylose resin (New England Biolabs). The resin was washed with Buffer P, and MBP SAS-6 was eluted with Buffer P + 30 mM maltose. Eluted proteins were centrifuged in a TLA120.2 rotor (100,000 rpm, 10 min, 4°C) to clear any insoluble products and flash frozen in liquid nitrogen until use. MBP SAS-5(203-404) 6xHis was purified as above.

**Pull-down assays**
Proteins were subjected to Superose 12 gel filtration, concentrated in 100K MWCO Amicon Ultra concentrators (Millipore), and dialyzed extensively into 25 mM HEPES/KOH pH 7.4, 100 mM KCl, 20 mM imidazole, 10 mM β-mercaptoethanol, 10% sucrose (w/v). MBP-tagged full length or 1-389 SAS-6 were incubated for 30 mins at 4°C with either bare Ni-NTA agarose, or Ni-NTA agarose pre-bound to MBP SAS-5(203-404) 6xHis. Final concentrations in each reaction were 1 μM SAS-6 and 2.5 μM SAS-5. Beads were washed three times and resuspended in SDS-PAGE sample buffer. Equal volumes were loaded onto 10% SDS-PAGE gels for analysis.

ACKNOWLEDGEMENTS

We thank the Caenorhabditis Genetics Center for strains. This work was supported by a grant from the National Institutes of Health (R01-GM074207) to K.O.. M.M.L. was supported by the University of California, San Diego Genetics Training Program (T32 GM008666) and Cancer Genetics Training Program. K.O. and A.D. receive salary and additional support from the Ludwig Institute for Cancer Research.

Chapter 3, in full, is in preparation for submission. It may appear as Molly M. Lettman, Valeria Viscardi, Yao Wong, Arshad Desai, and Karen Oegema. Three Distinct Regions of SAS-6 are Required for its Recruitment to the Mother Centriole. The dissertation author was the primary researcher and author of this paper.
ABBREVIATIONS

RR = RNAi-Resistant; C-Tail = C-terminal tail of SAS-6; RNAi = RNA interference; PCM = pericentriolar material; PLK4 = polo-like kinase 4
Figure 3.1 (A-C). The SAS-6 C-terminal tail is required for centriole assembly. (A) A C-terminal tail of varying length follows the coiled coil in SAS-6 homologs from different species. KEN boxes, which target SAS-6 for proteasomal degradation in some species (Strnad et al., 2007), are also marked. Coiled coil domains were predicted using Paircoil2 (McDonnell et al., 2006) with a 28 residue window. (B) Schematic of SAS-6 C-terminal truncations used to make SAS-6::GFP-expressing C.elegans strains. Truncations below the dotted line failed to support centriole assembly in vivo. (C) A western blot of lysates prepared from worms carrying transgenes directing the expression of GFP fusions with wild type (WT) SAS-6 or the indicated C-terminal SAS-6 truncations was probed with antibodies to SAS-6 (top) and α-tubulin as a loading control (bottom). The two right lanes are lysates from worms carrying the indicated C-terminal truncations of the sas-6::GFP transgene that are homozygous for a deletion of the endogenous sas-6 locus (ok2554, Δ).
Figure 3.1 (D-G). The SAS-6 C-terminal tail is required for centriole assembly. (D) Graph plotting embryonic lethality following sas-6 RNAi in worms carrying the indicated SAS-6::GFP encoding transgenes. Error bars = SD of per worm lethality, N = number of worms, n = number of embryos scored. (E) Schematic of the phenotypic outcome of a failure in centriole assembly. In the first division, a normal bipolar spindle, with centrosomes organized by the two sperm centrioles, is observed. Both daughter cells then inherit only a single sperm-derived centriole, resulting in monopolar spindles in the second division. (F) Graph of the percentage of monopolar cells observed following sas-6 RNAi in worms containing the indicated SAS-6::GFP encoding transgenes. (G) Western blot of worm lysates probed with antibodies to SAS-6 (top) and α-tubulin as a loading control (bottom). Extracts from strains carrying C-terminal truncations of SAS-6::GFP transgenes with or without RNAi to deplete endogenous SAS-6 were compared.
Figure 3.2. Compositional bias, rather than a specific sequence, is required in the SAS-6 C-terminal tail. (A) Schematic of SAS-6::GFP transgenes to examine the importance of sequence features of the SAS-6 C-terminal tail. (B) Graph of the percentage of monopolar cells observed following sas-6 RNAi in worms containing the indicated SAS-6::GFP encoding transgenes. (C) Graph plotting embryonic lethality following sas-6 RNAi in worms carrying the indicated SAS-6::GFP encoding transgenes. Plotted as in Fig. 1D.
Figure 3.3. The SAS-6 C-terminal tail is required for recruitment to the site of new centriole assembly. (A) The molecular pathway for C. elegans centriole assembly. (B) Schematic of assay used to monitor recruitment of SAS-6::GFP to the site of new centriole assembly. Embryos expressing SAS-6::GFP transgenes and mCherry::SPD-2 were depleted of endogenous SAS-6 by RNAi and mated to males with unlabeled centrioles (containing no SAS-6::GFP). mCherry::SPD-2 was used to mark centrioles and GFP coincident with RFP was quantified. Males also expressed mCherry::histone to confirm that embryos were the product of mating. (C) Graph of the percentage of monopolar cells observed following sas-6 RNAi in worms containing the indicated SAS-6::GFP encoding transgenes at 21°C or 16°C. Monitoring embryos at 16°C slows down the cell cycle and allows the visualization of SAS-6::GFP recruitment separate from assembly into the cartwheel. The 21°C data is reproduced from Figure 1F for comparison. (D) Kinetic recruitment profiles for WT SAS-6::GFP and GFP fusions with SAS-6-I154E and SAS-6 1-388. SAS-6::GFP is normally recruited to centrioles concurrent with cartwheel formation during S-phase and excess SAS-6::GFP that is not incorporated into the cartwheel is lost during mitosis. SAS-6-I154E is recruited during S-phase, but no cartwheel is built and all signal is lost during mitosis. SAS-6 1-388 is not recruited to the site of new centriole assembly.
Figure 3.4. Models for the role of the SAS-6 C-terminal tail in centriole assembly. Schematic of possible mechanisms which explain the requirement for the SAS-6 C-terminal tail for localization to the site of new centriole assembly.
**WT SAS-6 387-492**
KLSMENLIEKIAHYRAQRFSAPSGLPGLQTGTLNRLTSFKPVGLPGHPTYGANLNSRTPFRDNTTLNFQNSTIATPHAFRFNSQLIADETTGSSVTNTPPAQR

**387-492 region, 409-424 shuffle (Fig. 2 mutant II)**
KLSMENLIEKIAHYRAQRFSGSTPRGPALPLTNLPQGLTPSVKPVGLPGHPTYGANLNSRPFRDNTTLNFQNSTIATPHAFRFNSQLIADETTGSSVTNTPPAQR

**387-492 region, 425-492 shuffle (Fig. 2 mutant III)**
KLSMENLIEKIAHYRAQRFSGSTPRGPALPLTNLPQGLTPSQEGKPTNNTLNPTGRQNSRIFSRPPVFPTLDTQSTVAAYNAAPSLFTLTNDPSNHTIFAHTRSRIFSRPPVFPTLDTQSTVAAYNAAPSLFTLTNDPSNHTIFAHTRSRIFSRPPVFPTLDTQSTVAAYNAAPSLFTLTNDPSNHTIFAHTRRS

**387-492 region, 425-492 random (non SAS-6 sequence) (Fig. 2 mutant VII)**
KLSMENLIEKIAHYRAQRFSGSTPRGPALPLTNLPQGLTPQRTVDAEVGKRPHYSDEEVQKTATGLQAISLRSFMPKRKLSDLARPQIKEVSHEGVKTKASNL

**387-492 region, 409-492 random (non SAS-6 sequence) (Fig. 2 mutant VIII)**
KLSMENLIEKIAHYRAQRFSNEQTLTMSINQMLRFDVDAEVGKRPHYSDEEVQKTATGLQAISLRSFMPKRKLSDLARPQIKEVSHEGVKTKASNL

| BLACK | WT SAS-6 sequence |
| RED | WT SAS-6 sequence re-ordered ‘shuffled’ |
| PURPLE | non-SAS-6 sequence ‘random’ |

**Figure S3.1. Sequences of SAS-6 C-terminal tail mutants used in Fig. 3.2.**
Figure S3.2. The SAS-6 C-terminal tail is not required for binding to ZYG-1 or SAS-5 and does not affect oligomerization of SAS-6 through its N-terminus. (A) Schematic of known SAS-6 interactions that the C-terminal tail might modulate. (B) Results of a yeast two-hybrid assay between full length kinase defective ZYG-1 (K41M) and either full length SAS-6 or the SAS-6 coiled coil (residues 181-415). Assays with empty vector controls are also shown. (C) Coomassie stained gel comparing the ability of control beads and beads coated with purified SAS-5 (aa 203-404) to pull down purified full length or 1-389 SAS-6. (D) Schematic of the experiment used to compare the ability of full length or 1-389 SAS-6 to oligomerize through its N-terminal domains. Oligomerization of SAS-6 dimers generates a shift to larger size that can be detected by gel filtration. This shift is prevented by the I154E mutation, which prevents interactions between the SAS-6 N-terminal domains. (E) (left panels) Coomassie stained gels showing the purified WT and I154E variants of full length or 1-389 SAS-6 used in the self-assembly assay. (right panels) Proteins were loaded onto a gel filtration column, and elution was monitored by plotting refractive index versus elution volume. WT full length and 1-389 SAS-6 both elute earlier than their I154E mutant counterparts in a gel filtration assay in 150 mM NaCl (arrowheads mark elution peaks), suggesting that both can oligomerize through their N-termini.
Figure S3.3. The SAS-6 C-terminal tail is required for cartwheel assembly. (A) Maximum intensity projection confocal images of metaphase embryos expressing GFP fusions with WT or 1-424 SAS-6 generated as described in Fig. 3B. Scale bar=10µm. Insets magnified 3.4-fold. (B) Levels of WT and 1-424 SAS-6::GFP fusions at mitotic centrosomes were quantified during the 300s prior to cytokinesis, when the cartwheel has assembled and the amount of centriolar SAS-6 is nearly constant (see Figure 3C). All values were normalized to the average mitotic fluorescence intensity of WT SAS-6::GFP expressing embryos. Only the 47% of 1-424 SAS-6::GFP expressing embryos that fail centriole assembly were included in the quantification. Error bars represent the standard error of the mean, n=number of data points. In embryos that fail centriole assembly following depletion of endogenous SAS-6 by RNAi, SAS-6 1-424::GFP fails to target to centrosomes, suggesting that it does not get incorporated into the cartwheel.
Table S3.1. Average amino acid composition of SAS-6 C-tails (from *N.gruberi*, *T.brucei*, *L.major*, *C.reinhardtii*, *C.elegans*, *D.melanogaster*, *A.mellifera*, *D.rerio*, *H.sapiens*) compared to the average amino acid composition of all proteins (from UniProtKB/Swiss-Prot protein knowledgebase release 2012_07 statistics)

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Table S3.2. *C. elegans* strains, continued.

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Table S3.3. Oligos used for dsRNA production.

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Chapter 4. Conclusions and Future Directions

Centrioles have fascinated cell biologists for over a century. The conspicuous location of centrioles at the center of the spindle poles has long driven investigation into their function as centrosome organizers. More recently, their double life in cilia formation has begun to be appreciated.

The intricate symmetrical structure of centrioles has motivated intense investigation into how these organelles are built. The first step of centriole assembly is construction of the cartwheel. The cylindrical cartwheel is built from a stack of wagon wheel-like plates. Each of these plates contain an inner ring called the central hub and nine radially symmetric rod-like structures, called spokes, emanating outward. Around this cartwheel cylinder, a nine-fold symmetric outer wall of stabilized microtubules is built. The outer microtubule wall then extends past the cartwheel to reach the final defined length of the centriole. Finally, two sets of nine-fold symmetric appendages decorate the distal end of the centriole.

My graduate work has focused on trying to understand the molecular mechanisms of the first step of centriole assembly: construction of the centriolar cartwheel. When this dissertation work was initiated, the proteins involved in centriole assembly were just beginning to be identified. Several proteomic studies have expanded the list of proteins that localize to centrosomes to the hundreds. Surprisingly few, however, function in the step of cartwheel formation. One seminal study early in my graduate school career
allowed us, for the first time, to link the function of centriolar proteins to distinct steps in the assembly pathway. This study identified the proteins SPD-2, ZYG-1, SAS-6 and SAS-5 as necessary for the step of cartwheel formation (Pelletier et al., 2006). This list has remained unchanged and my thesis work has helped to shed light onto the mechanisms by which these proteins build the cartwheel (Figure 4.1).

SAS-6 is the main structural component of the centriolar cartwheel. SAS-6 family members contain an N-terminal globular domain, a long central coiled coil, and a C-terminus that lacks predicted secondary structure. SAS-6 dimerizes through its long central coiled coil and interactions between the N-terminal globular domains drive formation of a curved ring. This curved ring constitutes the central hub of the cartwheel and the coiled coils build the spokes. The interactions between the N-termini dictate the nine-fold symmetry of the cartwheel, and the coiled coil spokes contribute to the centriole diameter.

Although SAS-6 alone can oligomerize to form structures that resemble the centriolar cartwheel in both overall dimensions and symmetry (Kitagawa et al., 2011; van Breugel et al., 2011), several lines of evidence suggest that other proteins likely play a role in the regulation and stabilization of this assembly. First, the assemblies made by SAS-6 in vitro can deviate from the strict nine-fold symmetry found in the cartwheel in vivo and can result in incompletely closed structures. Second, the affinity of the interaction between SAS-6 N-termini is quite low (~100 µM), suggesting this interaction could be
facilitated by other factors or high local concentrations *in vivo*. Lastly, modeling of the crystal structure of the SAS-6 N-terminus suggests that N-terminal interactions could drive the formation of helical structures rather than closed circular cartwheel plates, suggesting that other factors could help enforce a plate-like structure.

Additionally, the process of centriole assembly is highly regulated such that it occurs precisely once per cell cycle and only in the vicinity of a mother centriole. Overexpression of SAS-6 or prevention of its degradation can result in the formation of too many centrioles per mother centriole in a variety of systems (Peel et al., 2007; Strnad et al., 2007). A desire to understand the regulation of centriole assembly, by understanding regulation of SAS-6 assemblies, was the driving force behind this thesis work.

**Uncoupling SAS-6 localization and cartwheel assembly**

Previously, it was not possible to distinguish between perturbations that disrupt SAS-6 localization and assembly of the centriolar cartwheel *per se*, thus it was difficult to gain mechanistic insight into how different mutations blocked centriole assembly. The dissertation work presented here uncouples these events for the first time, allowing further dissection of the discernible steps of centriole assembly.

Interestingly, different SAS-6 mutants that are able to localize to the mother centriole, but unable to build the centriolar cartwheel show a remarkably similar pattern of localization (to ~40% WT levels with similar
dynamics, Fig. 2.2, 2.3), suggesting the presence of a local, concentrated pool of SAS-6 from which the centriolar cartwheel is built. As the total SAS-6 levels at centrioles are higher when cartwheel assembly succeeds, the pool is likely replenished as SAS-6 is removed from the pool to build the cartwheel. This local pool is then lost when the time for cartwheel assembly has passed as the cell enters mitosis, whether or not cartwheel assembly has succeeded.

**Recognition of three distinct sites on SAS-6 is required for its localization to the mother centriole**

I identified a direct binding interaction between ZYG-1 and the SAS-6 coiled coil. Further, using a novel screen for mutations that block binding to coiled coil domains, I identified SAS-6 point mutants that are unable to bind ZYG-1 and additional independent mutants that block binding to the previously identified binding partner of SAS-6, SAS-5 (Fig. 2.4). Surprisingly, the distinct interactions between the SAS-6 coiled coil and both ZYG-1 and SAS-5, as well as the C-terminus of SAS-6, whose sequence is poorly conserved and lacks predicted secondary structure, are each required for the localization of SAS-6 to the mother centriole (Fig. 2.5, 3.3). The requirement for three SAS-6 activities for its localization suggests that the recruitment of SAS-6 to the site of new centriole assembly to establish the local pool is a highly regulated process.

One can imagine several models of how these interactions might work together in SAS-6 localization. First, the three modes of SAS-6 recognition
could function as a coincidence detector: only when you satisfy restrictions imposed by all three recognition sites do you bring SAS-6 to the local pool. This would require simultaneous binding by both ZYG-1 and SAS-5 and input from the SAS-6 C-terminus. Interestingly, although the ZYG-1 and SAS-5 binding sites on the SAS-6 coiled coil are close together (~55 amino acids apart), only one ZYG-1 or SAS-5 binding site is necessary per SAS-6 dimer as SAS-6 mutants defective in either interaction can incorporate into the cartwheel if they heterodimerize with endogenous SAS-6. An alternative idea is that each region of SAS-6 important for its localization could act sequentially, possibly by modifying SAS-6 in some way to prepare it for binding to the next partner or for self assembly. As the ZYG-1 and SAS-5 binding sites on the SAS-6 coiled coil are very close to each other, a hand-off from SAS-5 to ZYG-1 is also possible. Inroads into these questions would likely require in vitro binding studies to determine if ZYG-1 and SAS-5 can simultaneously bind to SAS-6.

A major challenge of teasing apart how each of these SAS-6 activities contributes to SAS-6 localization and cartwheel formation is the undefined nature of the requirement of the SAS-6 C-terminus. It has been proposed that SAS-6 dimers can associate in an anti-parallel fashion to build a tetramer and that this tetramer is an auto-inhibited conformation that prevents SAS-6 from forming cartwheel-like assemblies ectopically (Qiao et al., 2012). One could imagine that the C-terminus plays a role in tetramer formation and that this influences SAS-6 localization. It will be important, going forward, to determine
if loss of the C-terminus of SAS-6 abrogates tetramer formation and to investigate how different conformations of SAS-6 influence binding to ZYG-1 and SAS-5.

*Regulating local pool of centriolar proteins key to preventing overduplication?*

A key question in the field of centriole assembly is the desire to understand how only a single centriole is built next to a mother centriole during each cell division. From the above data, clearly the localization of SAS-6 is a highly regulated process. This is important because stockpiles sufficient to build many centrioles are present in the cytoplasm. One mechanism for limiting the number of centrioles that are built per cell cycle must be regulating the amount of centriolar proteins that localize to mother centriole. This could maintain a local concentration high enough to nucleate the growth of one and only one centriole, then favor growth of this centriole over nucleation of another (Goehring and Hyman, 2012).

Then how is this local pool regulated? Previous experiments have shown that increasing the total levels of SAS-6 by overexpression or preventing its degradation can result in the formation of too many daughter centrioles per mother centriole (Peel et al., 2007; Strnad et al., 2007), suggesting that increasing cytoplasmic levels of SAS-6 can increase its recruitment to the site of new centriole assembly. Additionally, SAS-6 localization is likely influenced by the number of SAS-6 binding sites on the
mother centriole. As ZYG-1 localizes before SAS-6 and a direct physical interaction between ZYG-1 and SAS-6 is required for SAS-6 localization, I would predict that altering the number of ZYG-1 binding sites at the mother centriole would alter the recruitment of SAS-6 and perhaps suppress centriole assembly or promote overduplication. This could be easily investigated by determining the minimum region of ZYG-1 that is sufficient for its interaction with SAS-6 and tethering this ZYG-1 region to different proteins. In addition, this experiment could be performed in the context of SAS-6 mutants that cannot assemble the centriolar cartwheel and the ‘local pool’ of SAS-6 could be directly investigated.

The target of the kinase activity of ZYG-1/Plk4 remains unknown

ZYG-1 is the key kinase that triggers cartwheel assembly. The kinase activity of ZYG-1 is required for centriole assembly, and interestingly, it is required for cartwheel assembly per se, but dispensible for SAS-6 localization (Fig. 2.3). SAS-6 has been the long-suspected substrate of ZYG-1 because of the critical role for each of these proteins in cartwheel assembly. Phosphorylation of SAS-6 by ZYG-1, and its importance in centriole assembly, has also been reported in the literature (Kitagawa et al., 2009). Here we provide evidence that SAS-6 is unlikely to be the target of the kinase activity of ZYG-1 (Fig. 2.6, 2.7), reopening the mystery of the ZYG-1 target.

Only four proteins are known to be required for cartwheel assembly: SPD-2, ZYG-1, SAS-6 and SAS-5. As steps preceding cartwheel assembly
appear normal in the absence of ZYG-1 kinase activity (SAS-6 and ZYG-1 itself localize normally, Fig. 2.3), but cartwheel assembly fails, we suspect the most likely target of the kinase activity of ZYG-1 is SAS-5. A key future direction will be to investigate the possibility that SAS-5 is the critical ZYG-1 substrate. In vitro kinase assays with both SAS-6 and SAS-5 have proven largely uninformative as ZYG-1 weakly phosphorylates many sites. Thus in order to determine if any SAS-5 serines or threonines are key points of cartwheel assembly regulation, an in vivo approach seems more productive. As generating directly comparable wild type and mutant single-copy C. elegans transgenic strains is straightforward, a series of SAS-5 mutants should quickly illuminate the question as to whether SAS-5 is a likely kinase substrate. Further experiments on promising candidate sites, such as studies into genetic interactions with ZYG-1 mutants (as in Fig. 2.6) could link these sites to ZYG-1 activity.

**Final thoughts**

In the last decade, there has been a dramatic advance in our understanding of centriole assembly. We now know the identity of many/all of the critical players in the process and the identity of the main structural component of the cartwheel. My thesis work has begun to shed light onto the complex method by which assembly of the centriolar cartwheel is regulated. Limiting the local pool of SAS-6 at the site of centriole assembly by the mechanisms presented here is likely critical to limit centriole assembly to once
per cell cycle. We can now further dissect the process of centriole assembly into activities required for the localization of SAS-6 and its incorporation into the cartwheel. An imminent obstacle will be to further dissect the events leading up to, promoting, and regulating the localization of SAS-6. Further, we have re-opened the mystery of the target of the ZYG-1 kinase activity, which will be a very exciting avenue to explore.
Figure 4.1. Summary of new findings presented in this thesis. (A) The process of cartwheel assembly can now be further dissected into two steps: 1. SAS-6 localization and 2. SAS-6 incorporation into the cartwheel. (B) SAS-6 localization requires three distinct regions of SAS-6: interactions between the SAS-6 coiled coil and ZYG-1 and SAS-5, and the SAS-6 C-terminus. (C) SAS-6 incorporation into the cartwheel requires ZYG-1 kinase activity and interactions between the SAS-6 N-termini (Kitagawa et al., 2011; van Breugel et al., 2011).
References


