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Centriole Assembly Requires Both Centriolar and Pericentriolar Material Proteins

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Summary

Centrioles organize pericentriolar material to form centrosomes and also template the formation of cilia. Despite the importance of centrioles in dividing and differentiated cells, their assembly remains poorly understood at a molecular level. Here, we develop a fluorescence microscopy-based assay for centriole assembly in the 1-cell stage *C. elegans* embryo. We use this assay to characterize SAS-6, a centriolar protein that we identified based on its requirement for centrosome duplication. We show that SAS-6, a member of a conserved metazoan protein family, is specifically required for new centriole assembly, a result we confirm by electron microscopy. We further use the centriole assembly assay to examine the roles of three pericentriolar material proteins: SPD-5, the kinase aurora-A, and γ-tubulin. Our results suggest that the pericentriolar material promotes daughter centriole formation by concentrating γ-tubulin around the parent centriole. Thus, both centriolar and pericentriolar material proteins contribute to centriole assembly.

Introduction

Centrioles are eukaryotic organelles that have two divergent functions: (1) they organize centrosomes, cellular organelles that nucleate and anchor microtubules (Bornens, 2002; Rieder et al., 2001); and (2) they template the formation of cilia, microtubule-based projections that perform a variety of motile and sensory functions (Snell et al., 2004).

Centrioles are cylindrical structures, ~100–200 nm in diameter and ~100–400 nm in length (Marshall, 2001; Preble et al., 2000), composed of a 9-fold symmetric array of stabilized microtubules (singlet, doublet, or triplet, depending on organism and tissue). New centrioles usually form adjacent to pre-existing centrioles, which are thought to template their assembly (reviewed in Delattre and Gonczy, 2004). Electron microscopy has defined a morphological pathway for centriole assembly (Anderson and Brenner, 1971, and references therein). First, a disc of fibrous material forms adjacent to the proximal end of the parental centriole. Next, a "cartwheel," consisting of a set of 9-fold symmetric spokes connected to a central axis, appears within this material. Centriolar microtubules assemble at the tips of each of the cartwheel spokes, and the structure elongates to form the mature centriole. In dividing cells, parental centrioles template the formation of 1 centriole per cell cycle. However, in ciliated tissues, up to 8 centrioles can form simultaneously, generally in a radial array around the base of the parent centriole. New centrioles can also form in association with other structures, notably the deuterosome during ciliogenesis in vertebrates (Anderson and Brenner, 1971) and the blepharoplast during spermatogenesis in ferns (Mizukami and Gall, 1966).

In the context of the centrosome, centrioles recruit pericentriolar material (PCM) to form a focal body (Bobinnec et al., 1998) that nucleates and anchors microtubules. Centrioles therefore determine centrosome number (Mazia et al., 1960; Sluder and Rieder, 1985), and assembly of new centrioles is a prerequisite for centrosome duplication (Delattre et al., 2004). Studies of extracted centrosomes indicate that centrioles are tightly associated with a fibrous matrix that directs the recruitment of other PCM components including the microtubule nucleator γ-tubulin (Moritz et al., 1998; Schnackenberg et al., 1998). In addition to its role in nucleating and organizing microtubules, pericentriolar material may also play a role in centriole assembly. When centrosomes are destroyed by laser ablation in vertebrate somatic cells, foci of pericentriolar material reform first, and new centrioles subsequently assemble de novo within these foci (Khodjakov et al., 2002). Although this topic has received less attention, PCM proteins may also be involved in centriole assembly during ciliogenesis (Kubo et al., 1999).

The identification of proteins required for centriole assembly has been hindered by their essential role in cell division and their small size, which has necessitated the use of serial-section EM to confidently score centriole number. Nevertheless, a number of proteins have been identified that directly function in centriole assembly. These include centrin-2 in vertebrates (Salisbury et al., 2002); SAS-4 (Kirkham et al., 2003; Leidel and Gonczy, 2003), SPD-2 (Kemp et al., 2004; Pelletier et al., 2004), and the kinase ZYG-1 (O'Connell et al., 2001) in *C. elegans*; and Bld10p in *Chylamydomonas* (Matsuura et al., 2004). In organisms whose centrioles have doublet or triplet microtubules, δ- and ϵ-tubulin are also required for centriole assembly (reviewed in Dutcher, 2003). The PCM protein γ-tubulin has also been implicated in basal body assembly based on studies in *Paramecium* and

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Tetrahymena (Ruiz et al., 1999; Shang et al., 2002). However, the essential functions of γ-tubulin in cell growth and division have complicated analysis of its role in centriole assembly.

The *C. elegans* embryo is well suited for studies of centriole function. RNA-mediated interference (RNAi) can be used to generate oocytes containing cytoplasm >95% depleted of a targeted gene product independent of intrinsic protein turnover (Montgomery and Fire, 1998). Fertilization introduces a single centriole pair, whose capacity to duplicate and recruit pericentriolar material in the depleted cytoplasm can be assayed. The ease of RNAi combined with the sequenced genome has prompted genome-wide screens (Grant and Wilkinson, 2003) that have identified ~1600 genes required for embryonic viability, which would include proteins required for centriole assembly. These screens, as well as forward genetics and characterization of homologs of proteins identified in other systems, are rapidly generating a catalog of proteins required for centriole and pericentriolar material function.

To study centriole duplication in *C. elegans*, we developed a fluorescence microscopy assay for centriole assembly based on incorporation of a GFP fusion with the centriolar structural protein SAS-4. Using this assay, we show that SAS-6, a member of a conserved family of centriolar proteins, is specifically required for centriole assembly. We further use our assay to examine the role of the pericentriolar material in this process. Our results suggest that the pericentriolar material promotes centriole assembly by providing a localized source of γ-tubulin. Thus, both centriole components and pericentriolar material contribute to centriole assembly.

Results

Development of a Fluorescence-Based Assay for Centriole Assembly

During *C. elegans* fertilization, the sperm brings a pair of centrioles into the oocyte, which lacks centrioles. The sperm-derived centrioles separate and a new "daughter" centriole forms adjacent to each of them, so that by metaphase each spindle pole has a centrosome containing two full-length centrioles (Kirkham et al., 2003). Newly formed daughters remain closely associated with their parent and individual centrioles cannot be resolved by light microscopy until the centriole pair separates in late anaphase/telophase. Therefore, to specifically monitor the assembly of new centrioles, we used mating to introduce unlabeled sperm centrioles into oocytes expressing a GFP fusion with the centriolar structural component, SAS-4 (Figure 1A). Like α/β-tubulin (Kochanski and Borisy, 1990), SAS-4 is incorporated into centrioles during their assembly and does not subsequently exchange with the cytoplasmic pool (Kirkham et al., 2003; Leidel and Gonczy, 2003). Furthermore, SAS-4 is required for formation of the centriolar cylinder, and the amount of centriolar SAS-4 is proportional to at least one measure of centriole function (the amount of PCM that a centriole can recruit; Kirkham et al., 2003), suggesting that GFP:SAS-4 incorporation provides a reliable readout for the extent of centriole assembly. New centriole assembly was assayed in fixed embryos by immunofluorescence with antibodies to GFP (to detect newly formed centrioles) and SAS-4 (to detect all centrioles, both new and sperm derived). Since *C. elegans* is a hermaphrodite, false positives in this assay could result from self-fertilization with GFP-containing sperm. To avoid this problem, we constructed a strain expressing GFP:SAS-4 that also carries a temperature-sensitive mutation in *fem-1*, a gene required for sperm production (Nelson et al., 1978). Thus, all embryos produced by hermaphrodites raised at the nonpermissive temperature are derived from fertilization of GFP:SAS-4-expressing oocytes with wild-type unlabeled sperm.

We first used this assay to examine the timing of new centriole assembly in the *C. elegans* embryo (Figures 1B and 1C). Foci of GFP:SAS-4 first became detectable concomitant with the onset of chromosome condensation during the first mitotic division (Figure 1B). Classifying embryos into early, middle, and late prophase based on their DNA morphology (see Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/7/6/815/DC1/) revealed that both the number of centrioles with a detectable GFP signal (Figure 1C) and the intensity of the signal (Figure 1B) gradually increased concurrent with chromosome condensation during prophase. During this time interval, centrosomes also mature in preparation for mitosis, with both the amount of PCM surrounding the centrioles and the number of centrosomally organized microtubules increasing ~5-fold (Hannak et al., 2001). By late prophase, when the chromosomes were fully condensed, clear foci of GFP:SAS-4 were associated with both sperm centrioles in all embryos examined (Figures 1B and 1C). In anaphase/telophase, when the accumulated mitotic PCM disperses and the paired centrioles separate in preparation for another round of duplication, GFP:SAS-4 is clearly visible associated with the newly formed centriole at each spindle pole, while the other, sperm-derived centriole remains unlabeled, confirming that centriolar SAS-4 does not exchange with the cytoplasmic pool (Figure 1B). Thus, this assay allows us to use fluorescence microscopy to monitor the assembly and fate of new centrioles that form in the oocyte cytoplasm. Our analysis indicates that centriole assembly occurs concurrent with chromosome condensation and centrosome maturation, during the last 12 min of the ~40 min period between fertilization and metaphase of the first mitotic division.

Identification of Two Proteins Required for Centrosome Duplication

Previous work identified two *C. elegans* proteins specifically required for centrosome duplication, SAS-4 and the kinase ZYG-1 (Kirkham et al., 2003; Leidel and Gonczy, 2003; O'Connell et al., 2001). Depletion of either protein results in an apparently normal first division followed by assembly of monopolar spindles in both daughter cells during the second division. In a differential interference contrast (DIC) microscopy-based RNAi screen for genes required for cell division, we identified two additional genes, F35B12.5 and Y45F10D.9, whose inhibition results in spindle assembly defects first apparent during the second division. By analogy with *sas-4*, these genes were named *sas-5* and *sas-6*, respectively (reflecting their role in bipolar spindle assembly).

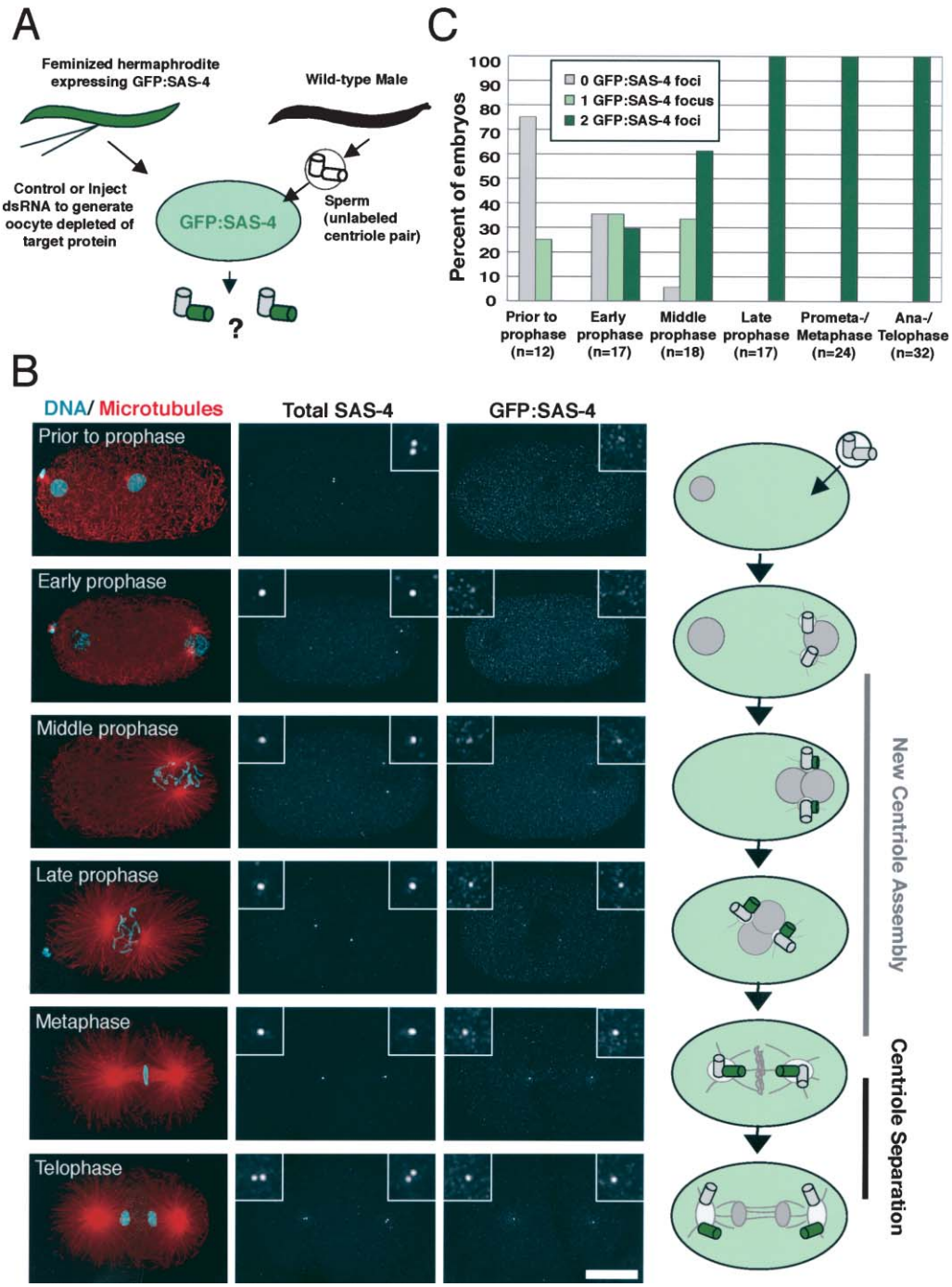

Figure 1. A Fluorescence Assay for Centriole Assembly

(A) Feminized hermaphrodites expressing a GFP fusion with the centriolar structural protein SAS-4 are mated with wild-type males. Fertilization introduces a pair of unlabeled centrioles (gray) into an oocyte containing GFP:SAS-4. Sperm centrioles separate, and assembly of new GFP-containing centrioles (green) adjacent to each of the sperm centrioles is scored. To test if specific proteins are required for centriole assembly, hermaphrodites are injected with dsRNA prior to mating to generate oocytes expressing GFP:SAS-4 that are depleted of the targeted protein.
(B) Centrioles assemble during prophase of the first mitotic division. Embryos resulting from mating feminized hermaphrodites expressing GFP:SAS-4 to wild-type males were fixed and stained for DNA, microtubules, SAS-4, and GFP. Schematics illustrate the progression of new centriole assembly suggested by incorporation of GFP:SAS-4. Scale bar equals 10 μm. Insets are magnified 3.5×.
(C) The number of new centrioles (foci staining for both SAS-4 and GFP) was quantified in embryos of the indicated cell cycle states.

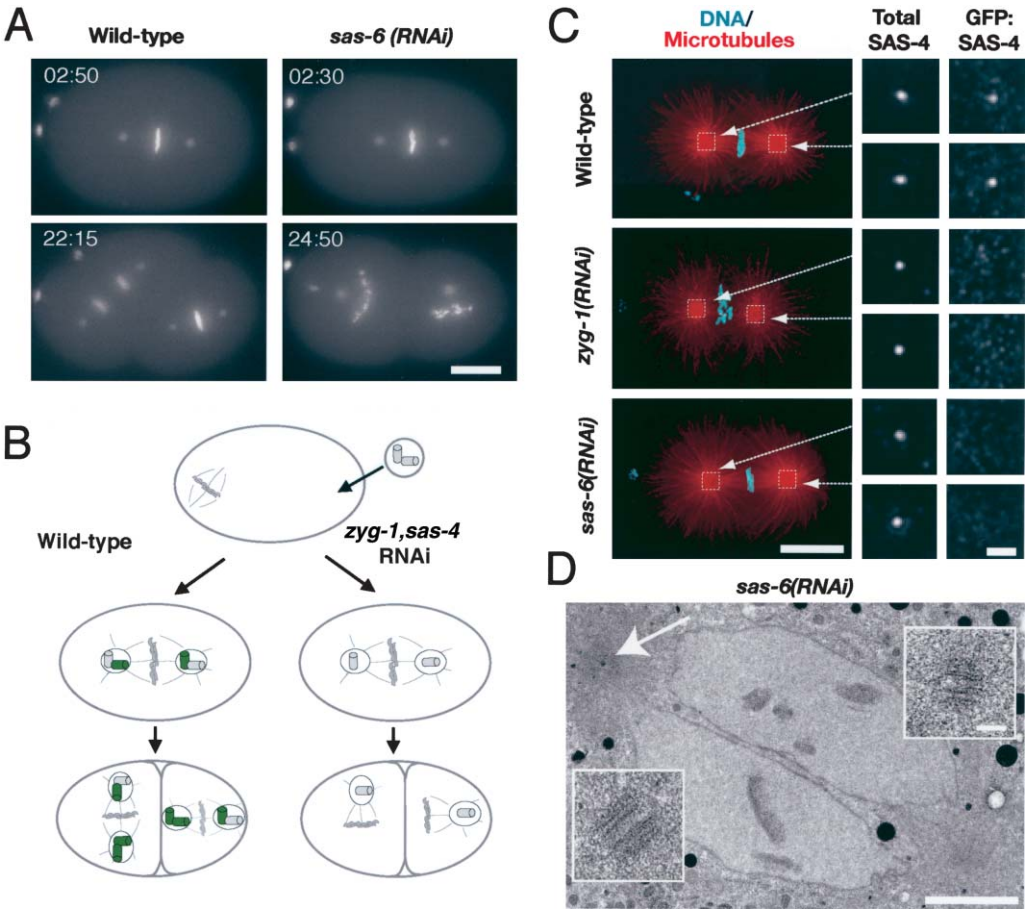

Figure 2. SAS-6 Is Specifically Required for Centriole Assembly

(A) Stills from time-lapse sequences of wild-type and *sas-6(RNAi)* embryos expressing GFP:histone and GFP:γ-tubulin (see also Supplemental Movies S1–S6). Times are minutes after nuclear envelope breakdown. Scale bar equals 10 μm.

(B) The centrosome duplication failure previously described for ZYG-1 and SAS-4-depleted embryos results from a failure of new centriole assembly (Kirkham et al., 2003; O'Connell et al., 2001).

(C) Results of centriole assembly assays performed on embryos depleted of SAS-6 or ZYG-1. Embryos were fixed and stained for DNA and microtubules (left), total SAS-4 (middle), and GFP:SAS-4 (right). Total SAS-4 and GFP:SAS-4 panels are higher magnification views of the indicated regions. Scale bars equal 10 and 1 μm, respectively.

(D) Correlative DIC/transmission EM confirms that each spindle pole in *sas-6(RNAi)* embryos contains only a single centriole (n = 5). Overview image illustrates the mitotic state of the chromatin. The position of a single centriole present in this section is indicated by an arrow. Insets are higher magnification views taken from sections containing the single centriole found at each spindle pole. Scale bars equal 2.5 μm and 100 nm, respectively.

To further analyze SAS-5 and SAS-6, we used RNAi to deplete them in embryos coexpressing GFP:γ-tubulin and GFP:histone to simultaneously monitor centrosomes and chromosomes (Figure 2A; Supplemental Figure S2A and Supplemental Movies S1–S3). In all *sas-5* and *sas-6(RNAi)* embryos filmed (n = 6 for each), bipolar spindles with levels of centrosomal γ-tubulin fluorescence similar to wild-type formed during the first mitotic division. However, during the second division, monopolar spindles with only a single focus of γ-tubulin fluorescence were observed in each daughter cell. We also analyzed depleted embryos coexpressing GFP:histone and GFP:β-tubulin to examine the microtubule cytoskeleton (Supplemental Movies S4–S6). Spindle assembly appeared identical to wild-type during the first mitotic division in both *sas-5* and *sas-6(RNAi)* embryos. However, as the embryos entered their second division, each daughter cell assembled only a single centrosomal microtubule aster. These asters increased normally in size during mitotic entry and monopolar spindles were formed. Depletion of either SAS-5 or SAS-6 therefore results in a specific defect in centrosome duplication identical to that observed in embryos depleted of ZYG-1 or SAS-4. SAS-5 was independently discovered in a mutational screen, and a characterization of this protein was recently published (Delattre et al., 2004). In Supplemental Figure S2 we present our analysis of this protein, which confirms and extends this prior study. Here, we focus on the previously uncharacterized SAS-6.

SAS-6 Is Specifically Required for Centriole Assembly

Serial-section transmission EM of *sas-4(RNAi)* and *zyg-1* mutant embryos has shown that their centrosome duplication defects result from a failure of centriole assembly (Kirkham et al., 2003; O'Connell et al., 2001). During the apparently normal first division in these embryos, each

pole is organized by a centrosome containing a single sperm-derived centriole instead of the normal centriole pair (Figure 2B). Since each daughter cell inherits only one centriole, only one centrosome can form, resulting in monopolar spindles in the second mitotic division. The phenotype in *sas-6(RNAi)* embryos could result from a similar failure of centriole assembly. However, other defects could also give rise to this phenotype. For example, new centrioles may assemble and separate from the sperm-derived centrioles normally but fail to recruit pericentriolar material to organize a new centrosome. Alternatively, centriole separation may be defective, so that the sperm-derived and newly assembled centrioles remain together, forming a single centrosome during the second mitotic division.

To distinguish between these possibilities, we used our fluorescence centriole assembly assay. Double-stranded RNA (dsRNA) was injected into feminized hermaphrodites prior to mating (Figure 1A) to generate oocytes expressing GFP:SAS-4 that were depleted of SAS-6 or ZYG-1. We examined embryos at stages between late prophase and metaphase of the first mitotic division, when all wild-type embryos have foci of GFP:SAS-4 associated with both sperm centrioles (Figure 1C; n = 41). In ZYG-1-depleted embryos, each centrosomal aster contained a focus of SAS-4, corresponding to a single sperm centriole, but no associated foci of GFP:SAS-4 were detected (n = 22; Figure 2C). SAS-6-depleted embryos similarly failed to form GFP:SAS-4 foci, either associated with the sperm centrioles or elsewhere in the embryo cytoplasm (n = 26 embryos; Figure 2C).

To confirm that no centrioles formed in the absence of detectable SAS-4 incorporation, we performed correlative DIC/serial-section transmission EM on *sas-6(RNAi)* embryos (Figure 2D). Fertilized embryos were filmed by DIC microscopy until metaphase of the first embryonic mitosis, when they were rapidly fixed by laser permeabilization in the presence of glutaraldehyde. Serial-section transmission EM was used to count the number of centrioles at each spindle pole. While wild-type embryos contain two centrioles at each spindle pole in metaphase (Kirkham et al., 2003), *sas-6(RNAi)* embryos contained only a single centriole at each pole (n = 5). Thus, SAS-6 is specifically required for new centriole assembly, as is also the case for ZYG-1 (O'Connell et al., 2001), SAS-4 (Kirkham et al., 2003; Leidel and Gonczy, 2003), and SAS-5 (Delattre et al., 2004; see also correlative DIC/serial-section transmission EM in Supplemental Figure S2C). Furthermore, the agreement between the results of our fluorescence assay and the analysis of embryos depleted of ZYG-1, SAS-5, and SAS-6 by serial-section EM validates the use of our assay for monitoring centriole assembly.

SAS-6 Localizes to Centrioles

To determine if SAS-6 acts at centrioles, we examined its localization using affinity-purified antibodies. Embryos were also stained for SAS-4, which was previously localized to centrioles by immuno-EM (Kirkham et al., 2003). SAS-6 colocalized with SAS-4 to small focal structures in the center of the centrosome throughout the embryonic cell cycle (Figure 3A). SAS-6 also colocalized with SAS-4 in sperm (Figure 3B). To confirm that SAS-6 localizes to centrioles, we performed immuno-EM on embryos from wild-type hermaphrodites prepared by high-pressure freezing/freeze substitution. In sections containing centrioles, gold particles were observed associated with the centriolar cylinders (Figure 3C). We also performed immuno-EM for SAS-5 and found that it too localizes to the centriolar cylinders (Supplemental Figure S2D). The combination of light microscopy and EM analysis indicates that SAS-6, like SAS-4 and SAS-5, is a bona fide centriolar protein.

Centriole assembly occurs primarily in prophase (Figure 1C), but ZYG-1, which is required for centriole assembly, was previously reported to localize to centrioles only transiently between metaphase and telophase (O'Connell et al., 2001). To resolve this apparent contradiction, we re-examined ZYG-1 localization. Using our antibodies, we could detect ZYG-1 staining at centrioles throughout the cell cycle (Figure 3A). This localization was observed with two different antibodies as well as in a transgenic line expressing GFP:ZYG-1 (data not shown). However, unlike SAS-4 and SAS-6, no ZYG-1 staining was observed in sperm (Figure 3B). In summary, ZYG-1, like SAS-4, SAS-5, and SAS-6, localizes to centrioles at a time consistent with its role in centriole assembly.

SAS-6 Is a Member of a Conserved Family of Centriolar Proteins

SAS-6 is a 56 kDa protein with no obvious sequence motifs other than a centrally located coiled-coil domain. Database searches using the N-terminal, coiled-coil-free region of SAS-6 identified human DKFZp761A078 and *Drosophila* CG15524 as potential SAS-6 homologs (Figure 4A). Interestingly, the putative human homolog was present in the proteomic catalog of the mammalian centrosome, recently defined using mass spectrometry (Andersen et al., 2003). To test whether HsSAS-6 localizes to centrioles, we transiently transfected a construct directing expression of a YFP fusion. YFP:HsSAS-6 localized to small foci in the center of the PCM (defined by γ-tubulin staining), consistent with centriolar localization (Figure 4B). Thus, SAS-6 is a member of a widely conserved centriolar protein family.

Centrosome Maturation Is Not Required for New Centriole Assembly

Our analysis of GFP:SAS-4 incorporation (Figures 1B and 1C) indicates that centrioles assemble concurrent with the recruitment of additional PCM during mitotic entry (centrosome maturation). SPD-2, a protein that localizes to both centrioles and the PCM, has recently been shown to be required for both centrosome maturation and centriole assembly (Kemp et al., 2004; Pelletier et al., 2004), suggesting a link between these two processes. To determine if the recruitment of additional PCM during centrosome maturation is required for centriole assembly, we examined embryos depleted of the aurora-A kinase, AIR-1. Like SPD-2, AIR-1 is required for centrosome maturation (Hannak et al., 2001). In embryos depleted of either protein, sperm centrioles acquire a small amount of PCM following fertilization, but the dramatic accumulation of additional PCM that normally accompanies mitotic entry fails to occur (Figures 5A and

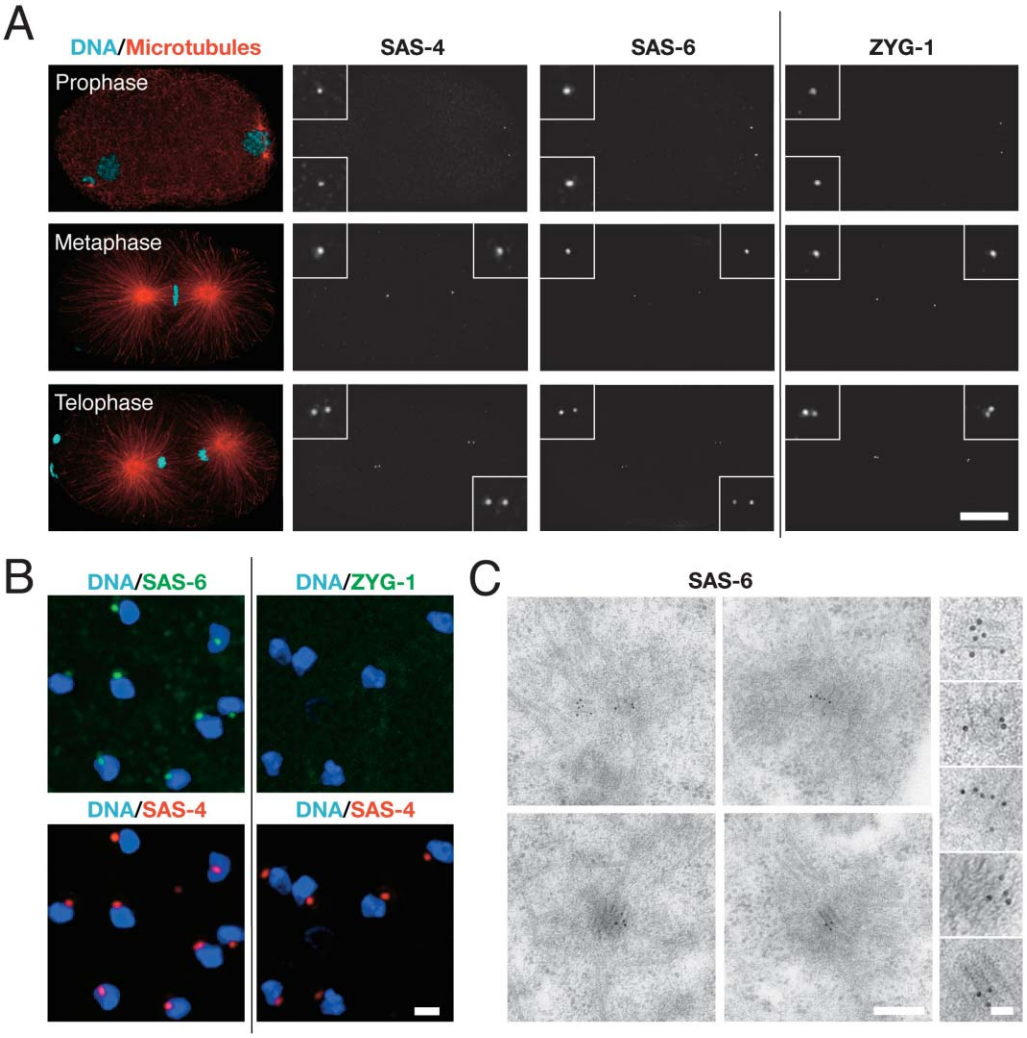

Figure 3. SAS-6 and ZYG-1 Colocalize with SAS-4 at Centrioles throughout the Cell Cycle

(A) Fixed embryos were stained for DNA, microtubules, SAS-4, and either SAS-6 or ZYG-1. DNA/microtubule, SAS-4, and SAS-6 images are from the same embryos. The ZYG-1 panels are from different embryos at similar cell cycle stages. Scale bar equals 10 μm. Insets are magnified 3.5×.

(B) SAS-6, but not ZYG-1, colocalizes with SAS-4 to a single focus on the nuclear periphery in mature sperm. Scale bar equals 1 μm.

(C) SAS-6 localizes to centrioles by immunoelectron microscopy. Scale bar equals 200 nm, insets 50 nm.

5B; Hannak et al., 2001; Pelletier et al., 2004; Kemp et al., 2004). We examined embryos in late prophase/prometaphase of the first mitotic division, which can be identified following even severe perturbations of centrosome function (see Experimental Procedures). At this stage, GFP:SAS-4 incorporation is observed associated with all sperm centrioles in wild-type embryos (Figure 1C). As expected, no foci of GFP:SAS-4 were detected in *spd-2(RNAi)* embryos ($n = 20$), either associated with the sperm centrioles or elsewhere in the cytoplasm. In contrast, GFP:SAS-4 incorporation occurred normally in AIR-1-depleted embryos (Figure 5C; $n = 11$), despite the failure of centrosome maturation. From these results, we can conclude that the mitosis-specific accumulation of PCM is not required for centriole assembly.

A Role for the Pericentriolar Material in Centriole Formation

Although PCM levels do not increase during mitotic entry in AIR-1-depleted embryos, sperm centrioles do acquire a basal level of PCM following fertilization. To determine whether this basal level contributes to centriole assembly, we analyzed embryos depleted of the pericentriolar material protein SPD-5 (Hamill et al., 2002). SPD-5 is an essential component of the PCM matrix; in *spd-5(RNAi)* embryos, no γ-tubulin or other pericentriolar material components accumulate around the centrioles at any cell cycle stage (Figure 6A; Hamill et al., 2002). In *spd-5(RNAi)* embryos, new centriole assembly did occur but was highly abnormal (Figure 6B). In wild-type embryos, both sperm centrioles are associated

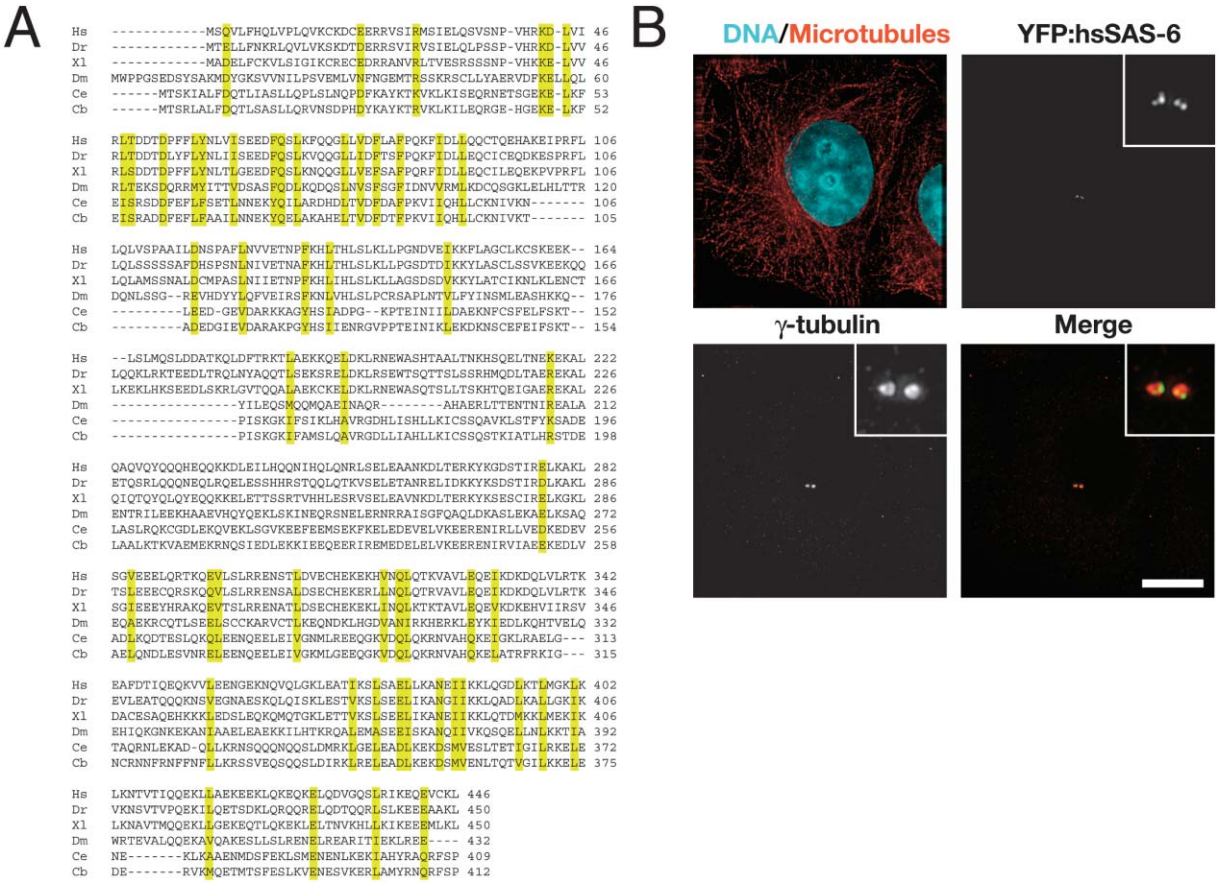

Figure 4. SAS-6 Is a Member of a Conserved Metazoan Centriolar Protein Family

(A) Partial sequence alignment of putative SAS-6 homologs. Conserved residues are highlighted in yellow. Abbreviations and accession numbers (in parentheses) of protein sequences: Hs, *Homo sapiens* (NP_919268.1); Dr, *Danio rerio* (NP_998603.1); Xl, *Xenopus laevis* (AAH70784.1); Dm, *Drosophila melanogaster* (NP_651756.1, CG15524-PA); Ce, *Caenorhabditis elegans* (NP_502660.1); Cb, *Caenorhabditis briggsae* (CAE74596.1).
(B) YFP:HsSAS-6 expressed in T98G human cultured cells localizes to two small foci within the larger PCM positive for γ-tubulin. Scale bar equals 10 μm. Insets magnified 4×.

with a focus of GFP:SAS-4 by late prophase/prometaphase, indicating that they have initiated the formation of a daughter centriole (Figure 1C). In contrast, only 29% of *spd-5(RNAi)* embryos at this stage contained two GFP:SAS-4 foci. In 43% of embryos, only a single GFP:SAS-4 focus was present, and in 29% of embryos no GFP:SAS-4 foci were detected, indicating that both new daughter centrioles failed to initiate assembly (Figure 6B). Thus, when the assembly of pericentriolar material around the sperm centrioles is blocked by depletion of SPD-5, new daughter centrioles fail to initiate ~50% of the time.

The partial failure to initiate new centriole assembly in *spd-5(RNAi)* embryos is in contrast to the complete block observed following depletion of ZYG-1, SAS-5, SAS-6, and SPD-2. To address whether residual centriole assembly might be due to inadequate depletion, we performed quantitative Western blotting and immunofluorescence. Western blots of injected worms revealed that total SPD-5 protein levels were reduced to ~1.5% of that in wild-type (Figure 6C). Similarly, the amount of SPD-5 detected at centrosomes in depleted mitotic 1-cell stage embryos was reduced by more than 99% (0.23% of wild-type levels; n = 24 wild-type embryos, 30 *spd-5(RNAi)* embryos). Thus, the partial disruption of new centriole assembly likely reflects the complete loss-of-function phenotype for SPD-5.

γ-Tubulin Promotes the Initiation of Centriole Assembly

SPD-5 could have a direct role in centriole assembly distinct from its role in PCM formation. Alternatively, the centriole assembly defect in *spd-5(RNAi)* embryos could result from failure to recruit another PCM component that promotes centriole assembly. An attractive candidate for such a component is γ-tubulin, a specialized tubulin required for centrosomal microtubule nucleation (Hannak et al., 2002). To test this idea, we analyzed the role of γ-tubulin in centriole assembly. Surprisingly, the GFP:SAS-4 incorporation assay revealed a defect in γ-tubulin-depleted embryos that was remarkably similar to that in embryos depleted of SPD-5 (Figure 7A). In

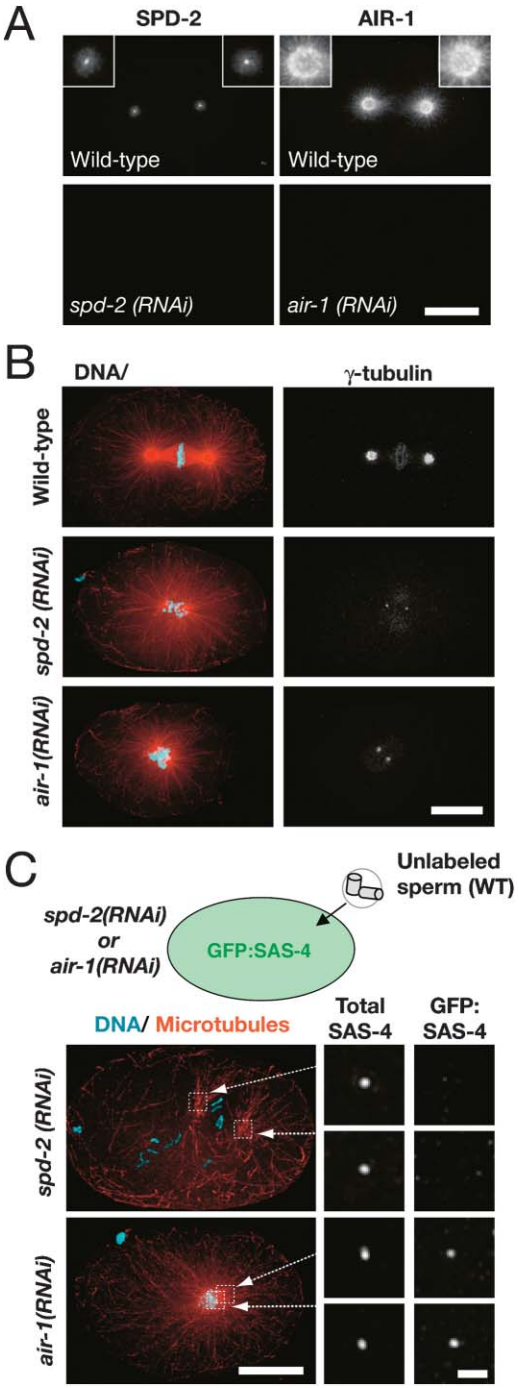

Figure 5. The Mitosis-Specific Accumulation of Pericentriolar Material Is Not Required for Centriole Assembly

(A) Wild-type, *spd-2(RNAi)*, and *air-1(RNAi)* embryos were fixed and stained for SPD-2 and AIR-1, as indicated. SPD-2 localizes to centrioles and weakly to the PCM. In contrast, AIR-1 localizes to the periphery of the centrosomes and extends out along astral microtubules. Both proteins are depleted beyond the detection limit following RNAi. Scale bar equals 10 μm. Insets magnified 2.5×.

(B) Wild-type, *spd-2(RNAi)*, and *air-1(RNAi)* embryos were fixed and stained for DNA and microtubules (left) and γ-tubulin (right). Mitotic centrosomes in depleted embryos remain small, reflecting a failure to recruit additional PCM during mitotic entry. Mitotic centrosomal microtubule asters also remain small and spindle assembly fails (Hannak et al., 2001; Kemp et al., 2004; Pelletier et al., 2004; see also Supplemental Movies S7–S9). Scale bar equals 10 μm.

(C) AIR-1 is not required for centriole assembly. Results of centriole assembly assays performed on embryos depleted of SPD-2 or AIR-1. Embryos were fixed and stained for DNA and microtubules (left), total SAS-4 (middle), and GFP:SAS-4 (right). Total SAS-4 and GFP:SAS-4 panels are higher magnification views of the indicated regions. Scale bars equal 10 and 1 μm, respectively.

32% of late prophase/prometaphase γ-tubulin-depleted embryos, no foci of GFP:SAS-4 were detected, indicating that both new daughter centrioles failed to initiate assembly. In 58% of embryos, only a single GFP:SAS-4 focus was present, and only 11% of embryos contained two GFP:SAS-4 foci (Figure 7B). These results indicate that new daughter centrioles failed to initiate assembly ~60% of the time in γ-tubulin-depleted embryos. A quantitative comparison of γ-tubulin and SPD-5-depleted embryos further underscores the similarity of the defect in the initiation of new centriole assembly (Figure 7B).

No γ-tubulin is detected around the sperm centrioles in SPD-5-depleted embryos (Figure 6A). Western blots revealed that γ-tubulin protein levels are unchanged following SPD-5 depletion (Figure 6C), indicating that γ-tubulin has not been destabilized, but rather fails to target to centrosomes. In contrast, depletion of γ-tubulin does not block PCM assembly, and centrosomal SPD-5 accumulates to normal levels in γ-tubulin-depleted embryos (Figure 7C). These results lead us to conclude that γ-tubulin is the key component of the PCM that is required to promote centriole assembly. The role of SPD-5 in centriole assembly is likely due to its role in targeting γ-tubulin to centrosomes.

Centrioles that Form in γ-Tubulin- or SPD-5-Depleted Embryos Are Aberrant

During our analysis of SPD-5- and γ-tubulin-depleted embryos, we found that when new centrioles did form, they often failed to remain in close proximity to their parent sperm-derived centrioles (Figure 7B). Premature separation of centriole pairs was observed in both SPD-5- and γ-tubulin-depleted embryos as early as late prophase, prior to nuclear envelope breakdown. By prometaphase/metaphase, 80% of new centrioles (n = 12/15) had separated from their parent in *spd-5(RNAi)* embryos and 60% (n = 6/10) had separated in *γ-tubulin (RNAi)* embryos. In contrast, paired centrioles in wild-type embryos never separate before late anaphase/telophase (n = 0/48; Figure 8A). The new centrioles (GFP-positive) in SPD-5- and γ-tubulin-depleted embryos also appeared to contain less total SAS-4 than the parental sperm-derived centrioles (Figure 8A). To determine if new centrioles were ultimately able to reach normal size, we quantified the ratio of total SAS-4 in the new centrioles to that in the old centrioles in telophase embryos, when centriole pairs have separated in wild-type (Figure 1). In control embryos, as well as in embryos depleted of AIR-1 (which is not required for the initiation of new centriole assembly; Figure 5B), this ratio is ~1, indicating that the new centrioles have reached full size. In contrast, in embryos depleted of SPD-5 or γ-tubulin, this ratio was ~0.6, suggesting that the new centrioles that do form fail to reach full size (Figures 8B and 8C).

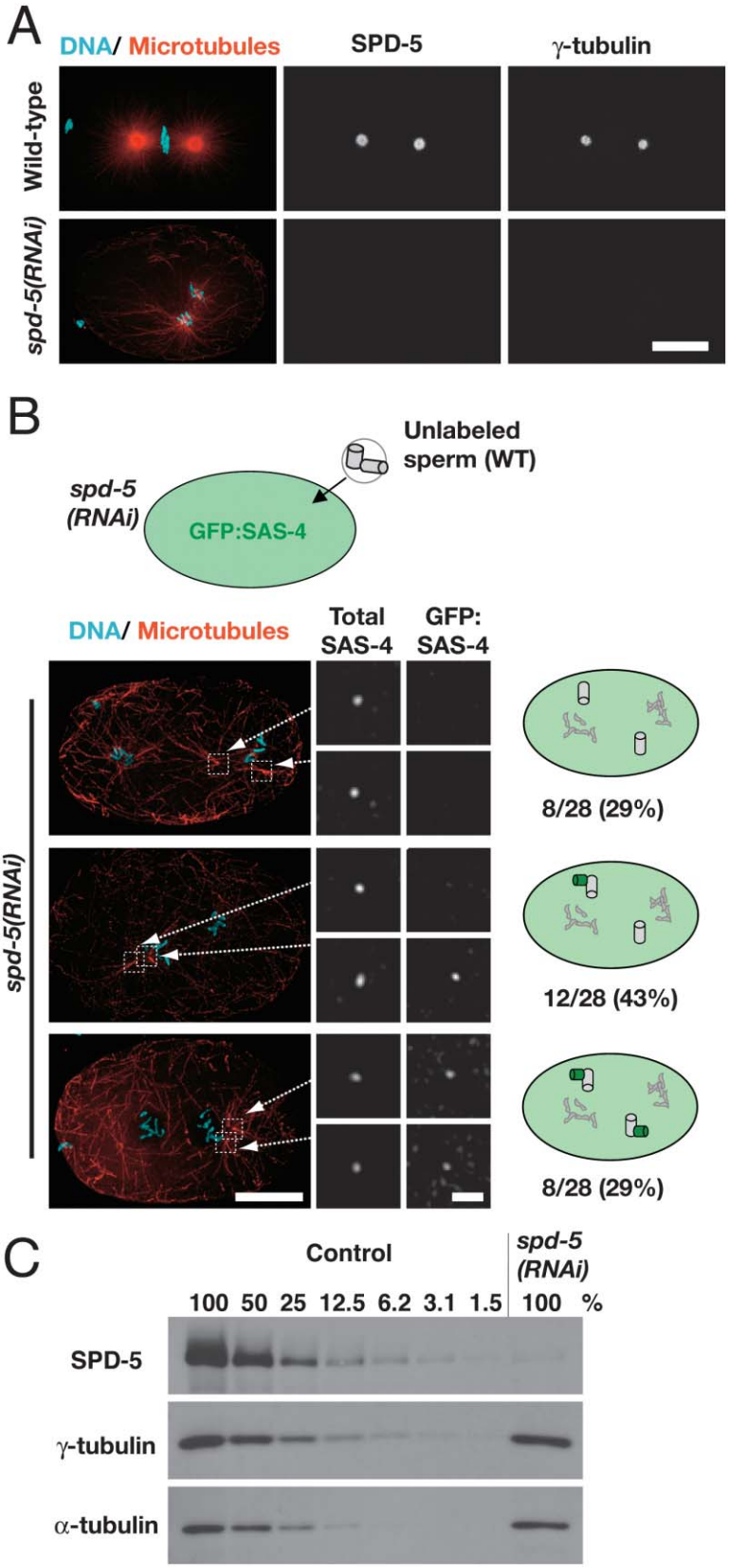

Figure 6. The Pericentriolar Material Protein SPD-5 Is Required for Proper Initiation of Centriole Assembly

(A) Wild-type and *spd-5(RNAi)* embryos were fixed and stained for DNA and microtubules (left), SPD-5 (middle), and γ-tubulin (right). SPD-5 colocalizes with γ-tubulin to the PCM in wild-type embryos. No γ-tubulin or other PCM components accumulate around the centrioles at any cell cycle stage in *spd-5(RNAi)* embryos (Hamill et al., 2002). Although weak microtubule asters form around the chromatin following nuclear envelope breakdown, no centrosomal microtubule asters form and spindle assembly fails (Hamill et al., 2002; see also Supplemental Movies S7 and S10).

(B) Results of centriole assembly assays performed on embryos depleted of SPD-5. Embryos were fixed and stained for DNA and microtubules (left), total SAS-4 (middle), and GFP:SAS-4 (right). Three classes of embryos, in which 0, 1, or 2 centrioles had duplicated, were observed as illustrated in the schematics to the right. Scale bars equal 10 and 1 μm (insets).

(C) Western blot comparing extract prepared from *spd-5(RNAi)* worms with serial dilutions of extract prepared from wild-type worms (numbers indicate percentage of amount loaded in 100% lane). SPD-5 is depleted to ~1.5% of wild-type levels. γ-tubulin levels are not affected by depletion of SPD-5. The same blot was probed for α-tubulin as a loading control.

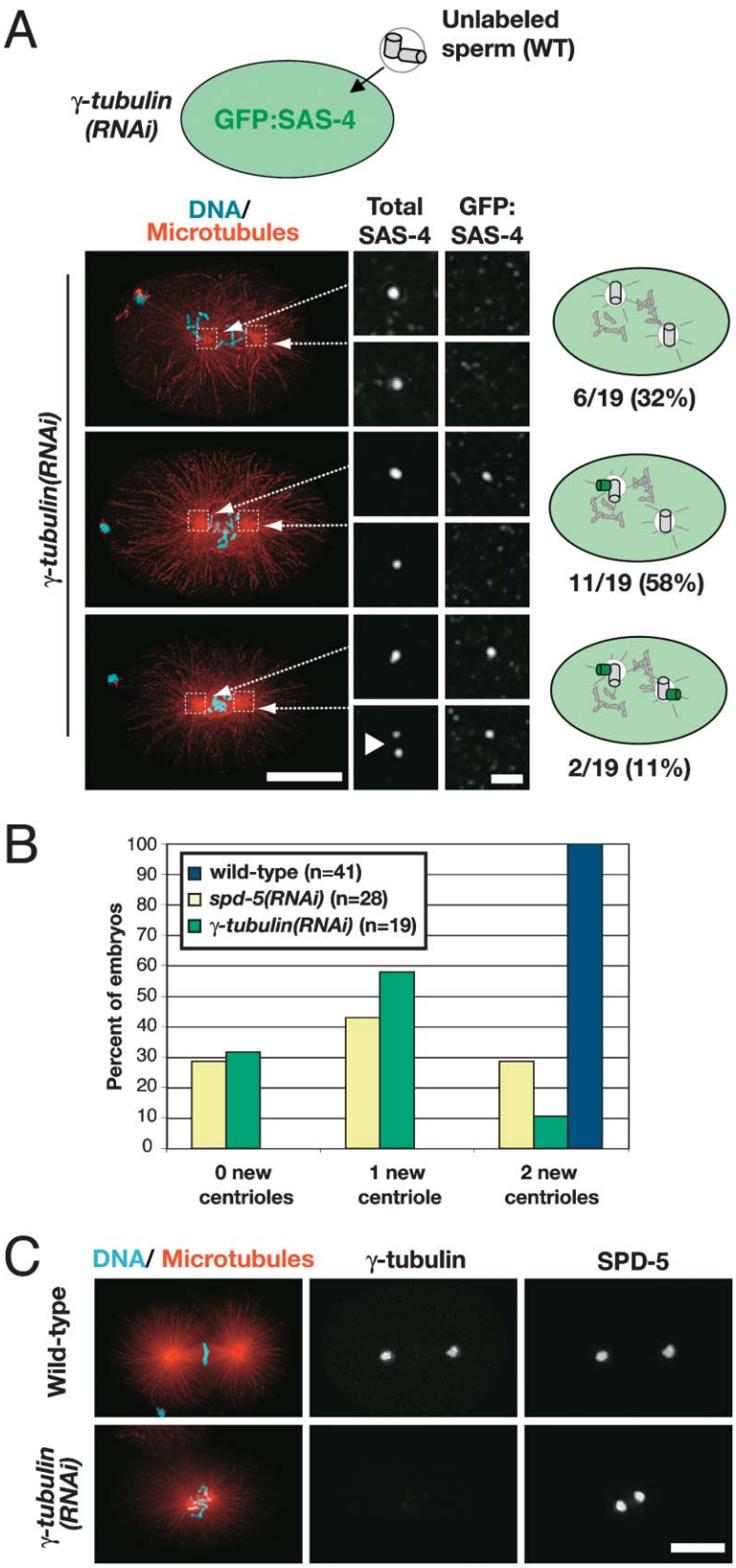

Figure 7. Depletion of γ-Tubulin Recapitulates the Centriole Assembly Defect Observed in *spd-5(RNAi)* Embryos

(A) Results of centriole assembly assays performed on embryos depleted of γ-tubulin. Embryos were fixed and stained for DNA and microtubules (left), total SAS-4 (middle), and GFP:SAS-4 (right). Three classes of embryos, in which 0, 1, or 2 centrioles had duplicated, were observed as illustrated in the schematics to the right. Arrowhead in lower panel highlights the premature separation of a new centriole from its parent (see Figure 8). Scale bars equal 10 and 1 μm (insets).

(B) The percentage of late prophase/prometaphase embryos that had formed 0, 1, and 2 new centrioles was quantified for wild-type, *spd-5(RNAi)*, and *γ-tubulin(RNAi)* embryos.

(C) Wild-type and *γ-tubulin(RNAi)* embryos were fixed and stained for DNA and microtubules (left), γ-tubulin (middle), and SPD-5 (right). Normal levels of SPD-5 assemble around centrioles in *γ-tubulin(RNAi)* embryos. Centrosomal microtubule asters form but centrosomal microtubule nucleation is severely compromised and spindle assembly fails (Hannak et al., 2002; see also Supplemental Movies S7 and S11).

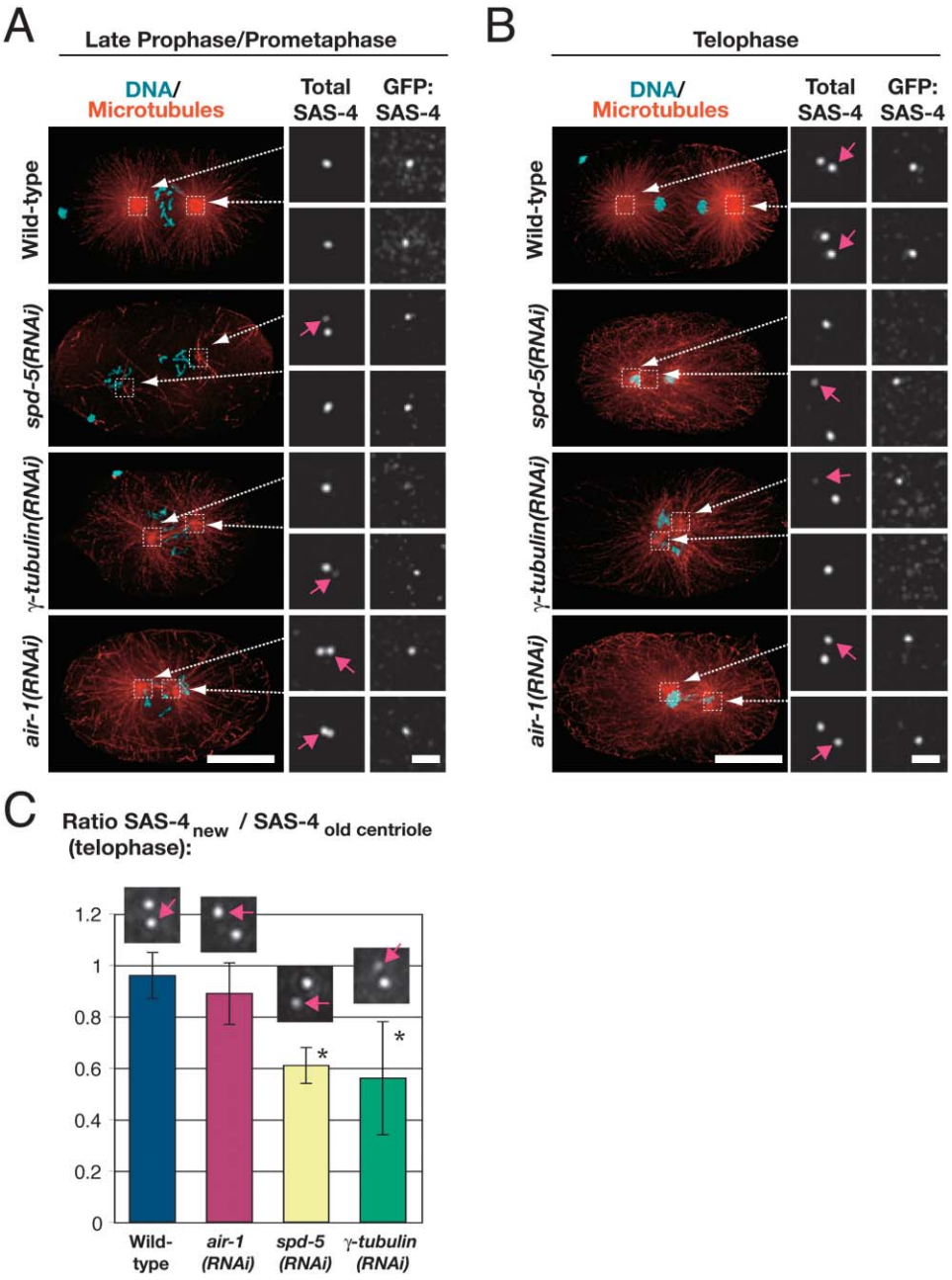

Figure 8. New Centrioles that Form in *spd-5* and γ-*tubulin(RNAi)* Embryos Are Aberrant

(A) In embryos depleted of SPD-5, γ-tubulin, or AIR-1, new centrioles often prematurely separate from their parent. Embryos were stained for DNA and microtubules (left), total SAS-4 (middle), and GFP:SAS-4 (right). New centrioles (containing GFP:SAS-4) are indicated with arrows in the total SAS-4 images. Scale bars equal 10 and 1 μm (insets).

(B) New centrioles that form in *spd-5* and γ-*tubulin(RNAi)* embryos fail to reach full size. Embryos in late anaphase/telophase (as assessed by DNA/microtubule morphology, see Experimental Procedures) were stained for DNA and microtubules (left), total SAS-4 (middle), and GFP:SAS-4 (right).

(C) Quantification of total centriolar SAS-4 fluorescence in anaphase/telophase-stage embryos. The ratio of total SAS-4 fluorescence on the new centriole (also positive for GFP) to that on the sperm-derived parental centriole was measured for 32 wild-type, 17 *spd-5(RNAi)*, 9 γ-*tubulin(RNAi)*, and 18 *air-1(RNAi)* centriole pairs. The average is plotted for each condition; error bars represent the SEM with a confidence interval of 0.95. Insets show representative images of SAS-4 staining (arrows point to the newly formed centrioles). Asterisks indicate statistically significant differences from wild-type (t test, $p < 0.001$).

New centrioles formed in γ-tubulin- and SPD-5-depleted embryos exhibit two distinct defects: premature separation from their parent and failure to reach full size. It seemed possible that the premature separation of immature new centrioles from their parent might prevent completion of their assembly. However, premature separation of centriole pairs was also observed following depletion of AIR-1 (Figure 8A; n = 11/14 centriole pairs

in prometaphase/metaphase embryos) without affecting the extent of SAS-4 incorporation (Figures 8B and 8C). Therefore, premature separation cannot explain the centriole assembly defect observed in SPD-5- or γ-tubulin-depleted embryos. In summary, our results indicate that depletion of SPD-5 or γ-tubulin results in a severe defect in centriole assembly. New centrioles fail to initiate ~50%–60% of the time, and the centrioles that do form contain less SAS-4 than wild-type, suggesting that they are incomplete or structurally defective.

Discussion

A Light Microscopic Assay for Centriole Assembly

Here we report the development of a fluorescence assay for centriole assembly based on incorporation of a GFP fusion with the centriolar protein SAS-4. The use of this assay is validated by our analysis of embryos depleted of four proteins shown to be required for new centriole formation by serial-section EM, ZYG-1 (O'Connell et al., 2001), SPD-2 (Pelletier et al., 2004), SAS-5 (Supplemental Figure S2), and SAS-6 (Figure 2). In all cases, no GFP:SAS-4 incorporation was detected in association with the sperm centrioles or elsewhere in the embryo.

We have used our ability to monitor centriole assembly at the light microscopic level to define a kinetic profile for this process during the first mitotic division of the *C. elegans* embryo. This allowed us to extend prior EM analysis, which showed the presence of full-length new centrioles at metaphase spindle poles (Kirkham et al., 2003). We have further used our fluorescence assay to characterize the role of the pericentriolar material in centriole assembly. This analysis relied on our ability to monitor centriole assembly following severe perturbations of centrosome structure and to find even defective centrioles that had prematurely separated from their parent. In addition, the assay allows analysis of large numbers of embryos, which would be prohibitively difficult using serial-section EM.

SAS-6 Is an Evolutionarily Conserved Protein Specifically Required for Centriole Assembly

Here, we report the identification of SAS-6, a centriolar component that, like ZYG-1 (O'Connell et al., 2001), SAS-4 (Kirkham et al., 2003; Leidel and Gonczy, 2003), and SAS-5 (Delattre et al., 2004, and this study), is specifically required for centriole assembly. To date, SAS-6 is the only member of this class of centriolar proteins to have identifiable homologs in other metazoans. We further show that the human homolog, HsSAS-6, is present on small foci within the PCM, consistent with localization to centrioles (Figure 4). Functional characterization of HsSAS-6 will be needed to determine if the essential role of SAS-6 in centriole assembly has also been conserved.

SAS-5 and SAS-6 colocalize with SAS-4 throughout the embryonic cell cycle and are associated with centrioles by immuno-EM (Figure 3 and Supplemental Figure S2). Unlike the other three proteins, ZYG-1 was previously reported to localize to centrioles only transiently between metaphase and telophase (O'Connell et al., 2001). In contrast, we found ZYG-1 to be localized to centrioles throughout the embryonic cell cycle (Figure 3). The previously reported antibody was raised against a ZYG-1 peptide (O'Connell et al., 2001), raising the possibility that this discrepancy is due to variations in epitope accessibility during the cell cycle. Thus, all four proteins specifically required for centriole assembly localize to centrioles at a time consistent with a role in their formation.

Partial depletion of SAS-4 results in defective centrioles that contain less SAS-4 and recruit proportionally less pericentriolar material (Kirkham et al., 2003). The high frequency with which intermediate phenotypes were observed following SAS-4 depletion is consistent with its proposed role as a structural component that is gradually incorporated during elongation. Similar depletions of ZYG-1 were reported to not result in intermediate phenotypes (Kirkham et al., 2003). However, we have recently found that assembly of defective centrioles that contain less than wild-type levels of SAS-4 and recruit less than wild-type amounts of γ-tubulin can also occur following partial depletions of SAS-5 and SAS-6 as well as ZYG-1, albeit at much lower frequencies than with SAS-4 (data not shown; see also Delattre et al., 2004). This may suggest that these three proteins play fundamentally different roles in centriole assembly (e.g., in initiation of centriole assembly rather than elongation) or simply reflect their different depletion kinetics.

Role of the Pericentriolar Material in Centriole Assembly

Our fluorescence assay made it technically feasible to perform a detailed analysis of the role of the PCM in centriole assembly. The timing of SAS-4 incorporation during the first mitotic division indicates that centriole assembly occurs concurrent with the recruitment of additional PCM during mitotic entry (centrosome maturation). However, centrioles form normally in *air-1(RNAi)* embryos in which centrosome maturation does not occur (Hannak et al., 2001), demonstrating that centriole assembly does not require the mitosis-specific recruitment of PCM. Conversely, centrosome maturation occurs normally in embryos depleted of ZYG-1, SAS-4, SAS-5, and SAS-6, in which centriole assembly fails (O'Connell et al., 2001; Kirkham et al., 2003; Leidel and Gonczy, 2003; Delattre et al., 2004; and this study). Thus, centriole assembly and the recruitment of additional PCM during centrosome maturation are independent events.

Although maturation is not required, our data do provide strong evidence for a role for the PCM in centriole assembly. We show that two proteins that localize to the PCM, SPD-5 (required for the formation of the PCM; Hamill et al., 2002) and γ-tubulin (required for centrosomal microtubule nucleation; Hannak et al., 2002; Strome et al., 2001), contribute to the formation of new centrioles. A role for γ-tubulin in centriole assembly is consistent with previous work in *Tetrahymena* and *Paramecium*, where inhibition of γ-tubulin expression caused defects in basal body duplication (Ruiz et al., 1999; Shang et al., 2002). The fact that we observe a defect in a rapidly dividing embryonic system in which γ-tubulin depletion does not halt cell growth or cell cycle progression (Hannak et al., 2002; Strome et al., 2001) strongly supports the idea that γ-tubulin has a direct role in centriole assembly.

Following depletion of γ-tubulin or SPD-5, new daughter centrioles fail to initiate ~50% of the time and the new centrioles that do form contain less SAS-4 than wild-type centrioles. The fact that centriole assembly is not completely blocked in our assay is consistent with previous work showing that older SPD-5-depleted embryos often contain multiple centrioles (Kemp et al., 2004; Pelletier et al., 2004). The partial defect in centriole assembly in SPD-5- and γ-tubulin-depleted embryos is in contrast to the complete block observed following depletion of SAS-4, SAS-5, SAS-6, ZYG-1, and SPD-2 (O'Connell et al., 2001; Kirkham et al., 2003; Leidel and Gonczy, 2003; Delattre et al., 2004; and this study). Previously, we have shown that our γ-tubulin RNAi conditions reduce the amount of centrosomal γ-tubulin to ~2% of wild-type levels (Hannak et al., 2002). Here we show that centrosomal SPD-5 is similarly depleted following SPD-5 RNAi (0.23% of wild-type levels). Although we cannot exclude the possibility that the residual centriole assembly that we observe results from a small amount of remaining protein, the interpretation we favor is that the partial defect in centriole assembly in SPD-5- and γ-tubulin-depleted embryos represents the complete loss-of-function phenotypes for these proteins.

What is the nature of the centriole assembly defect in γ-tubulin/SPD-5-depleted embryos? Our previous characterization of SAS-4 demonstrated that partial reduction of the level of centriolar SAS-4 results in the assembly of partial centrioles, as assayed by serial-section EM and by reduced ability to recruit PCM components such as γ-tubulin (Kirkham et al., 2003). This analysis strongly supports the idea that the centrioles containing reduced levels of SAS-4 that form in γ-tubulin/SPD-5-depleted embryos are either less than full length or structurally defective. One possibility is that γ-tubulin/SPD-5 contribute to the generation of a template that directs new centriole assembly. In depleted embryos, some centriole assembly occurs, but the resulting structures are abnormal. Alternatively, γ-tubulin/SPD-5 might normally function to accelerate the kinetics of centriole assembly. In this case, new centrioles may be structurally normal but fail to elongate to their normal length within the time constraints imposed by the rapid cell cycles of the early *C. elegans* embryo. Resolving this issue is an important future direction that will require structural analysis by 3D EM tomography.

One of our most intriguing findings is a role in centriole formation for SPD-5. In SPD-5-depleted embryos, all of the PCM components that have been tested, including γ-tubulin, fail to concentrate around the centrioles (Hamill et al., 2002; Kemp et al., 2004; Pelletier et al., 2004). One attractive possibility suggested by our data is that assembly of PCM around the parent centriole is required to generate a local concentration of γ-tubulin, which in turn promotes centriole formation. Consistent with this idea, depletion of γ-tubulin alone can recapitulate the centriole assembly defect seen in SPD-5-depleted embryos without preventing the recruitment of other PCM proteins.

Experimental Procedures

Worm Strains and Culture Conditions

Strains coexpressing GFP:histone and GFP:γ-tubulin (TH32), and GFP:histone and GFP:β-tubulin (XA3501), were described previously (Askjaer et al., 2002; Desai et al., 2003). The centriole assembly assay strain OD19 was obtained by mating BA17 and DP38 to generate a *fem-1(hc17)*, *unc-119(ed3)* strain. Males expressing GFP:SAS-4 (TH26; Kirkham et al., 2003) were mated to this strain, F2 individuals were singled, and a line homozygous for the temperature-sensitive female-sterile mutation *fem-1(hc17)* and positive for the GFP:SAS-4 transgene was identified. Strain genotypes are listed in Supplemental Table S1. Strains were maintained at 16°C (OD19) or 20°C (TH32, XA3501). For the SAS-4 incorporation assay, OD19 was shifted to 25°C as L4 larvae. L4 progeny of shifted worms were injected with dsRNA and mated to wild-type (N2 Bristol) males. OD19 worms raised to the L4 larval stage at 25°C remain female-sterile even if returned to the permissive temperature (16°C).

RNA-Mediated Interference

dsRNA was prepared as described (Oegema et al., 2001). DNA templates were prepared by using the primers listed in Supplemental Table S2 to amplify regions of genomic N2 DNA or specific cDNAs, as indicated. For depletion of γ-tubulin and AIR-1, the RNAi conditions described previously that led to penetrant depletion were used (Hannak et al., 2001, 2002). For all other depletions, L4 larvae were incubated at 16°C for 48 hr following dsRNA injection, prior to analysis of their embryos.

Live Imaging

Live imaging of embryos coexpressing GFP:γ-tubulin and GFP:histone was performed as described (Desai et al., 2003).

Immunofluorescence, Fixed Imaging, and Quantification of Centrosomal Fluorescence

Antibodies to γ-tubulin and AIR-1 were described previously (Hannak et al., 2001). Goat anti-GFP was a gift from David Drechsel (MPI-CBG, Dresden, Germany). Polyclonal antibodies against SAS-4 (residues 181–300), ZYG-1 (201–402), SAS-5 (1–210), SPD-2 (2–200), and SPD-5 (392–550) as well as peptide antibodies to the C-terminal 17 amino acids of SAS-5 and the C-terminal 19 amino acids of SAS-6 were raised, affinity-purified, and directly labeled as described (Oegema et al., 2001). DM1α (Sigma) was used at a dilution of 1:1000. All other antibodies were used at a concentration of 1 μg/ml. Embryos were fixed and processed for immunofluorescence as described (Oegema et al., 2001). 3D widefield data sets collected using a 100× 1.3 NA PlanApochromat lens on a DeltaVision microscope were computationally deconvolved and projected using SoftWorx software (Applied Precision). Quantification of centrosomal fluorescence was performed on deconvolved 3D image stacks as described (Hannak et al., 2002).

Staging of Fixed Embryos

Embryos were assigned to early, middle, and late prophase based on DNA and microtubule morphology (see Supplemental Figure S1). AIR-1, SPD-2, γ-tubulin, and SPD-5-depleted embryos were considered to be in late prophase of the first mitotic division if they had the expected number of fully condensed chromosomes, but the nuclear envelope was still intact (as assessed by exclusion of cytoplasmic GFP and tubulin fluorescence). Prometaphase/metaphase embryos had a similar DNA morphology to late prophase embryos, but the nuclear envelope had broken down. Prometaphase and metaphase embryos were grouped together because we could not distinguish between these stages in the absence of an intact spindle. Embryos at a stage analogous to anaphase/telophase in wild-type were identified by their DNA and microtubule morphology. Exit from mitosis was marked by aggregation of chromosomes and a marked increase in microtubule assembly in the vicinity of the chromatin. Separated sister chromatids were also frequently observed. DNA decondensation was further evident in late telophase embryos. This analysis was facilitated by comparison with aligned time-lapse sequences taken in the GFP:β-tubulin and GFP:histone line (see examples in Supplemental Movies S7–S11). Note that none of the molecular perturbations performed blocked cell cycle progression.

Correlative DIC/Transmission Electron Microscopy and Immunoelectron Microscopy

Correlative DIC/transmission EM and immuno-EM using the SAS-5 and SAS-6 peptide antibodies were performed as described (Kirkham et al., 2003).

Immunoblotting

Western blotting of *spd-5(RNAi)* and wild-type control worms was performed as described (Hannak et al., 2001).

Bioinformatics

BLASTP searches against the nonredundant database (May 2004 release) were performed using the NCBI-BLAST stand-alone software package. Multiple sequence alignments generated using ClustalX were manually refined.

Cloning of HsSAS-6 and Localization in Human Cells

Full-length HsSAS-6 was amplified by PCR from a HeLa cDNA library (provided by Jagesh Shah, UCSD, CA) and cloned into the expression vector pEYFP-C1 (Clontech). T98G human glioblastoma multiforme cells were maintained in DMEM supplemented with 10% FBS in a humidified 37°C, 5% CO_2 atmosphere. Transient transfections were performed by calcium phosphate precipitation. Cells were fixed for 10 min in methanol at −20°C 24 hr after transfection. For these experiments, γ-tubulin and microtubules were visualized using mouse (GTU-88, Sigma) and rat (YOL1/34, Abcam) monoclonal antibodies, respectively.

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Note Added in Proof

While this manuscript was in press, Raynaud-Messina et al. reported similar defects in centriole assembly following γ-tubulin depletion in *Drosophila* S2 cells (Raynaud-Messina, B., Mazzolini, L., Moisand, A., Cirinesi, A.M., and Wright, M. (2004). Elongation of centriolar microtubule triplets contributes to the formation of the mitotic spindle in γ-tubulin-depleted cells. J. Cell Sci. *117*, 5497–5507).