

# Finding treasures in frozen cells: new centriole intermediates

Susan K. Dutcher

## Summary

Centriole duplication has been an area of interest since the late 1800s when Boveri suggested that these structures were central organizers for mitosis and cell division. Two groups<sup>(1,2)</sup> have delineated a linear pathway for centriole assembly. In *C. elegans*, Pelletier and coworkers<sup>(1)</sup> have identified intermediates in the pathway using cryo-electron tomography. Surprising, the first intermediate is a hollow tube of 60 nm that increases in diameter and then elongates before acquiring microtubules. Similar structures have not been observed to date in other centrioles. *BioEssays* 29: 630–634, 2007. © 2007 Wiley Periodicals, Inc.

## Introduction

The centrosome of animal cells consists of a pair of centrioles and associated pericentriolar material. Centrioles are recognizable as cylinders of nine microtubules. In most mammalian cells as well as algae and protists, microtubules are present in a triplet arrangement and the centriole has an outer diameter of about 200 nm and a length of about 400 nm. In *Drosophila*, the microtubules are present as doublets in some tissues and as triplets in others.<sup>(3–5)</sup> In *C. elegans*, the microtubules are present as singlet microtubules.<sup>(6)</sup> From their early description in the late 1800s by light microscopy to their ultrastructural analysis by electron microscopy in the 1960s, questions about centriole structure and duplication have remained largely unanswered for over 50 years. First, their replication is tightly linked to other cell cycle events, but the mechanisms are only beginning to be unraveled. Second, their duplication is semi-conservative. Each old centriole is found at the pole of the mitotic spindle with a new centriole and thus the mitotic daughter cells will have an old and new centriole in the next G<sub>1</sub> of the cell cycle. Third, electron microscopic ultrastructure showed that the pair of centrioles has an orthogonal arrangement; the new centriole forms at right

angles to the old centriole during duplication. Finally, the events involved in duplication have been described in only a few organisms and the genes involved are unknown.

Recent studies in *C. elegans* have provided new details about the events and genes involved in duplication of the centrioles. Previous genetic analysis identified five genes that play a role in centriole duplication. They are *spd-2*, *zyg-1*, *sas-4*, *sas-5* and *sas-6*.<sup>(7–12)</sup> These gene products localize to centrioles and *spd-2*, *sas-4* and *sas-6* have homologs in other centriole-containing eukaryotes (Table 1). Mutations in each of these genes or RNA interference depletion of the gene products prevent centriole duplication. Other work has shown that Sas-6 is required for centriole duplication in mammals<sup>(11)</sup> and Sas-4 is required for centriole duplication in *Drosophila*.<sup>(13)</sup>

Centrioles in *C. elegans* are less complex than in mammalian cells by morphological criteria. The structure is smaller; they are only 150 nm by 100 nm and comprise nine singlet microtubules. The *C. elegans* embryo provides an excellent “test tube” for analyzing the events of centriole duplication. The features of the *C. elegans* embryo include the ability to deplete or nearly deplete a particular gene product in the first mitotic cell division by RNA interference. In addition, the events of the first division are highly reproducible and can be followed easily by light and electron microscopy.

As in many organisms, the *C. elegans* egg, which is arrested in prophase of meiosis I, has no centrioles, but has a large store of maternally contributed products. Upon fertilization in *C. elegans*, the sperm donates two intact centrioles. These centrioles must duplicate prior to the first mitotic division in order that a pair of centrioles will reside at each pole and will recruit pericentriolar material. To follow the formation of the new centrioles, a mating-based assay was used.<sup>(10,14,15)</sup> The assay relies on antibodies to the Sas-4 protein that mark the sperm-donated centrioles and the presence of a GFP-tagged gene in the cytoplasm of the egg from the hermaphrodite parent. This assay allows for the monitoring of new protein recruitment and incorporation via the GFP signal from the egg into both the old and new centrioles. Using these tools, two groups have independently demonstrated that the five genes mentioned above act in a linear pathway to promote centriole duplication.<sup>(1,2)</sup>

Previous work made two important observations. First, fluorescence recovery after photobleaching (FRAP)

Department of Genetics, Washington University School of Medicine,  
St. Louis, MO 63110. E-mail: dutcher@genetics.wustl.edu  
DOI 10.1002/bies.20594  
Published online in Wiley InterScience (www.interscience.wiley.com).

Abbreviations: Sas, spindle assembly abnormal; Zyg, zygote defective;  
Spd, spindle defective.

**Table 1.** *C. elegans* genes and human homologs

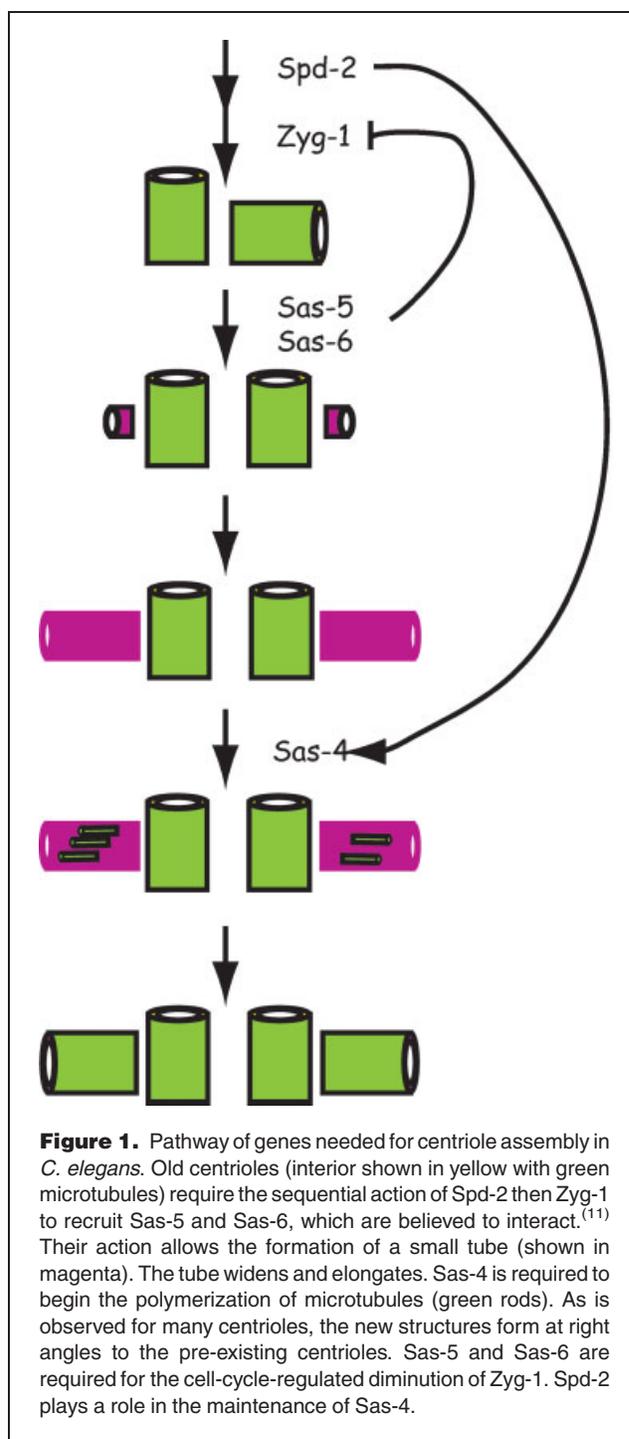
Gene Name	Homologs	Protein Features
<i>spd-2</i>	Cep192	Coiled Coil, T-complex protein domain (TcP10)
<i>zyg-1</i>	None	Kinase
<i>sas-4</i>	Human (CPAP or CenPJ), Drosophila	Coiled-coil
<i>sas-5</i>	None	Coiled-coil
<i>sas-6</i>	Hs-SAS6	Coiled-coil

experiments on GFP-labeled SAS-4 and SAS-6 showed that incorporation only occurs once per cycle and occurs after cytokinesis.<sup>(11)</sup> This result is consistent with centriole duplication occurring once per cell cycle. Second, using the mating-based assay with maternally contributed Sas proteins labeled with GFP, each of the *sas* genes showed a different behavior. Sas-4 was only recruited to new centrioles and remains associated with the structure. Sas-6 first appears on the old centriole and then on the new centriole. For both Sas-4 and Sas-6, the majority of the protein is associated with the centrioles. In contrast, Sas-5 protein shuttles between the centrioles and cytoplasm.

In the newly published papers “Centriole assembly in *Caenorhabditis elegans*” by Pelletier, O’Toole, Schwager, Hyman, and Müller-Reichert<sup>(1)</sup> and “Sequential protein recruitment in *C. elegans* centriole formation” by Delattre, Candard, and Gönczy,<sup>(2)</sup> the pathway for assembly and the assembly intermediates is determined.

Spd-2 and Zyg-1 are the first of the proteins to be recruited to the centrioles and this occurs prior to the completion of meiosis of the maternal nucleus (Fig. 1). Sas-5 and Sas-6 are recruited at the end of meiosis II and prior to the appearance of the pronucleus, which corresponds to the S-phase. Sas-4 is recruited while the cell is in S-phase (Table 2). Classical epistasis tests were performed using RNA interference to remove or reduce each of the five proteins from the egg cytoplasm, but not from the sperm centrioles. A simple pathway was obtained. Spd-2 acts first and is required for the recruitment of the other four proteins. Zyg-1, which is an atypical kinase, is required after Spd-2 is recruited. These two proteins are then required for the recruitment of Sas-5 and Sas-6, which are known to interact. With the presence of SAS-5 and SAS-6, the level of ZYG-1 decreases and SAS-4 is recruited (Fig. 1).

By light microscopy, the proteins become localized at the centrioles, as the newly fertilized zygote is finishing meiosis. However, conventional chemical fixation and thin-section microscopy failed to observe any intermediates in centriole assembly at these early time points. Pelletier and coworkers turned to high-pressure freezing and electron tomography.



High-pressure freezing has been well documented to allow the better preservation of cytoskeletal elements.<sup>(16)</sup> Electron tomography, which uses thick sections (200–400 nm), utilizes multiple images taken with different tilt angles as well as dual rotation of the sample. The information in these images is deconvolved using algorithms that allow computational

**Table 2.** Localization of centriole proteins

Protein name	Present on Sperm Centrioles	Timing of Appearance of Maternal Product	Appearance of Maternal Product on Old Centriole
Spd-2	+	Meiosis I	Yes
Zyg-1	–	Meiosis I	Yes
Sas-5	+	Meiosis II, first cell cycle	Yes
Sas-6	+	Meiosis II, first cell cycle	Yes
Sas-4	+	First cell cycle	No

sectioning of the images to several nanometers. Thus, electron tomography allows the detection of structures that have short lengths and the three-dimensional structure can be reconstructed from the images. A new high-pressure freezing apparatus was developed that allowed the investigators to follow a newly fertilized egg with the dissecting microscope and to freeze it at known times in the assembly process.

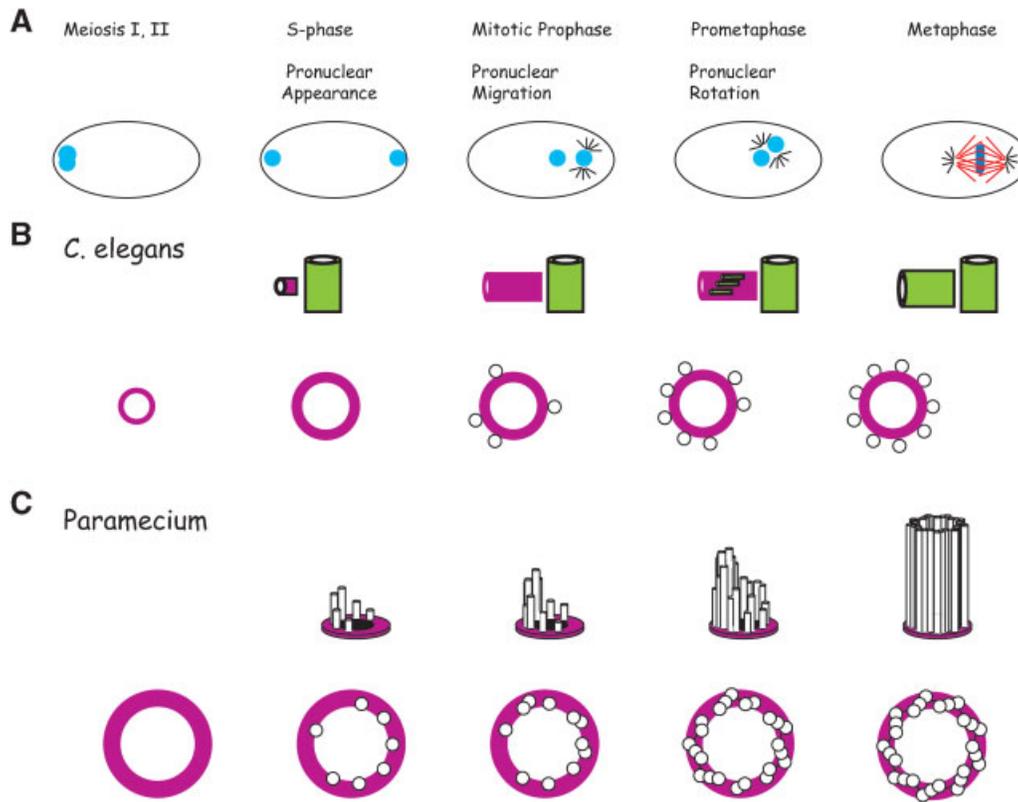
The first intermediate observed in the fertilized embryo is a hollow tube that is about 60 nm in length. The mature centrioles in *C. elegans* have a tube that lines the interior of the centriole. This small tube forms at right angles to the mature centriole. The tube elongates to a length of about 110 nm and increases in diameter by prophase of mitosis. The microtubules begin to assemble by prophase. The microtubule assembly does not routinely begin at the proximal or distal ends of the tube. By prometaphase, nine microtubules are present. Prior to the presence of an assembled microtubule, an electron-dense hook structure was observed in its place on the tube. The composition of these hooks is unknown. The assembly process takes about 8–10 minutes.

Using RNAi to deplete individual proteins, Pelletier and coworkers observed that no new centriole structures were observed in worms depleted for *zyg-1*, *sas5* and *sas-6*. The depletion of *sas-4*, which is needed at the last characterized step based on epistasis tests, still allows the formation of the central tube and the tube elongates. However, the tube fails to increase in width and no hooks or microtubules are observed. These *sas-4*-depleted tubes are unstable and not observed in older embryos. Thus, Pelletier and coworkers have characterized new intermediates in the centriole assembly pathway (Fig. 2A,B).

The assembly of basal bodies, which are similar in structure and can convert to centrioles during the cell cycle in many organisms, has been analyzed by conventional thin-section electron microscopy in *Paramecium*.<sup>(17)</sup> In *Paramecium*, thousands of basal bodies are present and their duplication is relatively synchronous, which allows many intermediates in the process to be identified. As discussed above for centrioles, the duplication occurs at right angles to the old basal body. The first sign is the appearance of a flat amorphous disc that was termed the generative disc. The disc has no radial structure and appears uniform in its morphology. It is the first structure to

form and is found at the proximal end of the developing basal bodies (Fig. 2C). Singlet microtubules appear and before all of the singlet microtubules are formed, the formation of doublet microtubules begins, and before all doublet microtubules have formed, the formation of triplet microtubules begins. The disc changes its structure as the basal body assembles and becomes hollowed out before the formation of the triplet microtubules to form a washer rather than a disc (Fig. 2C). High-pressure freezing and electron tomography has been used to examine basal bodies in *Chlamydomonas* a flagellated green alga. The images obtained by high-pressure freezing for fixation are compared to images obtained by conventional chemical fixation,<sup>(16,18–20)</sup> One noticeable difference is found. The frozen images show the presence of a ring of amorphous material (similar to the ring shown in purple in *Paramecium* in Fig. 2) that is located at the base or proximal end of the basal bodies. This ring in *Chlamydomonas* appears to show similar preservation properties to the tube in *C. elegans*. It is also possible that this amorphous ring material in *Chlamydomonas*, as well as the generative disc in *Paramecium*, may provide the same function as the tube in *C. elegans*.

Another structure that may play roles in mammalian centriole duplication is the 9-bladed pinwheel or cartwheel that is found near the proximal end of many basal bodies and centrioles. The *bld10* mutant in *Chlamydomonas* lacks a microtubule cylinder. Immunoelectron microscopy suggests that Bld10p is a constituent of the pinwheel.<sup>(21)</sup> It is not known if the ring of amorphous material is present in this mutant; the cells were examined following chemical fixation in which the ring is not stable. Like many of the gene products found by genetic analysis in *C. elegans*, and by mass spectroscopy studies of isolated mammalian centrosomes,<sup>(22)</sup> Bld10p is a coiled-coil protein. It has homologs in mammalian cells but no clear homolog in *C. elegans*. The pinwheel has been suggested to be responsible for the nine-fold symmetry of the centriolar microtubules. Given its absence in *C. elegans*, other determinants will need to be sought. How does the *C. elegans* tube or the amorphous material of *Chlamydomonas* and *Paramecium* specify the number of microtubules in the structure? Along a similar line, it still remains unclear why the new structures form at right angles to the old centriole. Perhaps further electron tomography will reveal radial



**Figure 2.** Centriole intermediates in *C. elegans* and *Paramecium*. **A:** Timing of the cell cycle events during centriole duplication. These events take 8–10 minutes.<sup>(1,2)</sup> The nuclei in the newly fertilized egg must complete meiosis and then initiate the first cell mitotic cycle. Black lines indicate the microtubule asters and red lines indicate the spindle microtubules. **B:** Centriole events in *C. elegans* uncovered by cryo-electron microscopy.<sup>(1)</sup> Events are shown diagrammatically in longitudinal sections in line 1 and in cross-sectional analysis in line two. The newly formed tube is shown in magenta and microtubules are shown in green in longitudinal sections and in black in cross-sectional analysis. The diameter of the tube is shown in proportion to the diameter of the generative disc in *Paramecium* in **C**. **C:** Basal body events in *Paramecium*.<sup>(17)</sup> The generative disc, which is likely to be equivalent to the amorphous ring in *Chlamydomonas*, is formed and subsequently hollowed out. Singlet microtubules are formed and then followed by doublet and triplet microtubules. Doublet microtubules are often formed before all of the singlet microtubules are made. Modified from Pelletier L, O’Toole E, Schwager A, Hyman AA, Muller-Reichert T 2006 Nature 444:619–623.

asymmetries that suggest an assembly site that allows for the recruitment of the Sas proteins.

**References**

- Pelletier L, O’Toole E, Schwager A, Hyman AA, Muller-Reichert T. 2006. Centriole assembly in *Caenorhabditis elegans*. Nature 444:619–623.
- Delattre M, Canard C, Gonczy P. 2006. Sequential protein recruitment in *C. elegans* centriole formation. Curr Biol 16:1844–1849.
- Callaini G, Whitfield WG, Riparbelli MG. 1997. Centriole and centrosome dynamics during the embryonic cell cycles that follow the formation of the cellular blastoderm in *Drosophila*. Exp Cell Res 234:183–190.
- McDonald K, Morpew MK. 1993. Improved preservation of ultrastructure in difficult-to-fix organisms by high pressure freezing and freeze substitution: I. *Drosophila melanogaster* and *Strongylocentrotus purpuratus* embryos. Microsc Res Tech 24:465–473.
- Moritz M, Braunfeld MB, Fung JC, Sedat JW, Alberts BM, et al. 1995. Three-dimensional structural characterization of centrosomes from early *Drosophila* embryos. J Cell Biol 130:1149–1159.
- Ward S, Argon Y, Nelson GA. 1981. Sperm morphogenesis in wild-type and fertilization-defective mutants of *Caenorhabditis elegans*. J Cell Biol 91:26–44.
- O’Connell KF, Maxwell KN, White JG. 2000. The *spd-2* gene is required for polarization of the anteroposterior axis and formation of the sperm asters in the *Caenorhabditis elegans* zygote. Dev Biol 222:55–70.
- O’Connell KF. 2002. The ZYG-1 kinase, a mitotic and meiotic regulator of centriole replication. Oncogene 21:6201–6208.
- O’Connell KF, Caron C, Kopish KR, Hurd DD, Kempfues KJ, et al. 2001. The *C. elegans* *zyg-1* gene encodes a regulator of centrosome duplication with distinct maternal and paternal roles in the embryo. Cell 105:547–558.
- Kirkham M, Muller-Reichert T, Oegema K, Grill S, Hyman AA. 2003. SAS-4 is a *C. elegans* centriolar protein that controls centrosome size. Cell 112:575–587.
- Leidel S, Delattre M, Cerutti L, Baumer K, Gonczy P. 2005. SAS-6 defines a protein family required for centrosome duplication in *C. elegans* and in human cells. Nat Cell Biol 7:115–125.
- Delattre M, Leidel S, Wani K, Baumer K, Bamat J, et al. 2004. Centriolar SAS-5 is required for centrosome duplication in *C. elegans*. Nat Cell Biol 6:656–664.

13. Basto R, Lau J, Vinogradova T, Gardiol A, Woods CG, et al. 2006. Flies without centrioles. *Cell* 125:1375–1386.
14. Kemp CA, Kopish KR, Zipperlen P, Ahringer J, O'Connell KF. 2004. Centrosome maturation and duplication in *C. elegans* require the coiled-coil protein SPD-2. *Dev Cell* 6:511–523.
15. Leidel S, Gonczy P. 2003. SAS-4 is essential for centrosome duplication in *C. elegans* and is recruited to daughter centrioles once per cell cycle. *Dev Cell* 4:431–439.
16. O'Toole ET, Giddings TH Jr, Dutcher SK. 2007. Understanding microtubule organizing centers by comparing mutant and wild-type structures with electron tomography. *Methods Cell Biol* 79:125–143.
17. Dippell RV. 1968. The development of basal bodies in paramecium. *Proc Natl Acad Sci USA* 61:461–468.
18. O'Toole ET, Giddings TH, McIntosh JR, Dutcher SK. 2003. Three-dimensional organization of basal bodies from wild-type and delta-tubulin deletion strains of *Chlamydomonas reinhardtii*. *Mol Biol Cell* 14:2999–3012.
19. Johnson UG, Porter KR. 1968. Fine structure of cell division in *Chlamydomonas reinhardtii*. Basal bodies and microtubules. *J Cell Biol* 38:403–425.
20. Ringo DL. 1967. Flagellar motion and fine structure of the flagellar apparatus in *Chlamydomonas*. *J Cell Biol* 33:543–571.
21. Matsuura K, Lefebvre PA, Kamiya R, Hirono M. 2004. Bld10p, a novel protein essential for basal body assembly in *Chlamydomonas*: localization to the cartwheel, the first ninefold symmetrical structure appearing during assembly. *J Cell Biol* 165:663–671.
22. Andersen JS, Wilkinson CJ, Mayor T, Mortensen P, Nigg EA, et al. 2003. Proteomic characterization of the human centrosome by protein correlation profiling. *Nature* 426:570–574.