The Origin of the Second Centriole in the Zygote of *Drosophila melanogaster*

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ABSTRACT Centrosomes are composed of two centrioles surrounded by pericentriolar material (PCM). However, the sperm and the oocyte modify or lose their centrosomes. Consequently, how the zygote establishes its first centrosome, and in particular, the origin of the second zygotic centriole, is uncertain. *Drosophila melanogaster* spermatids contain a single centriole called the Giant Centriole (GC) and a Proximal centriole-like (PCL) structure whose function is unknown. We found that, like the centriole, the PCL loses its protein markers at the end of spermiogenesis. After fertilization, the first two centrioles are observed via the recruitment of the zygotic PCM proteins and are seen in *asterless* mutant embryos that cannot form centrioles. The zygote's centriolar proteins label only the daughter centrioles of the first two centrioles. These observations demonstrate that the PCL is the origin for the second centriole in the *Drosophila* zygote and that a paternal centriole precursor, without centriolar proteins, is transmitted to the egg during fertilization.

CENTRIOLES, in the cytoplasm and basal bodies at the plasma membrane, are conserved microtubule-based organelles essential for cell division and cilium formation (Nigg and Raff 2009). Centrioles are essential for fertilization, development, and animal physiological functions (Nigg and Raff 2009). In the newly fertilized egg (*i.e.*, zygote), a centriole normally functions by recruiting pericentriolar material (PCM) and becoming the primary centrosome (Delattre and Gonczy 2004). This centrosome, in the zygote, acts as a microtubule-organizing center and nucleates the astral microtubules that mediate the migration of the female and male nuclei toward each other (Callaini and Riparbelli 1996).

A centriole forms by one of two pathways. In the "duplication pathway," a pre-existing centriole acts as a scaffold to ensure that only a daughter centriole is formed per cell cycle. However, the pre-existing centriole does not appear to impart structural information to the daughter (Rodrigues-Martins *et al.* 2007). In the "*de novo* pathway," a centriole forms without a pre-existing centriole and forms more than two centrioles. This pathway occurs when multiple centrioles are required in a cell or in the unusual situation where pre-existing centrioles are absent (Uetake *et al.* 2007).

Most resting cells have two centrioles. A cell preparing to divide duplicates its centrioles and consequently has four centrioles; each mother/daughter centriole pair forms a centrosome at opposite poles of the cell. Having precisely two centrioles before commitment to cell division and four centrioles during mitosis is particularly critical for proper cell division and an organism's development (Fukasawa 2007). Having no centrioles interferes with the proper orientation of the spindle axis (Basto *et al.* 2006), while too many centrioles results in an increase in aneuploidy and defects in cilium formation (Basto *et al.* 2008; Mahjoub and Stearns 2012). Because a cell requires two centrioles to function, the zygote is expected to require two centrioles. In animals, during oogenesis centrioles are lost, and therefore, oocytes lack centrioles and do not contribute any centrioles to the zygote (Sun and Schatten 2007). Instead, it has been reported that in many animals centrioles are inherited by the zygote from the sperm (Sun and Schatten 2007). However, in many other animals, a single functional centriole is observed. Humans and most mammalian sperm have only a single centriole because during the last phase of spermatogenesis the spermatid centrioles are modified and degraded in a process known as centrosome reduction (Manandhar *et al.* 2005). In sea urchins, frogs, and *Caenorhabditis elegans*, the sperm provides two centrioles (Longo and

Anderson 1968; Felix *et al.* 1994; Leidel 2005). The origin of this second zygotic centriole is uncertain. To help explain these uncertainties, four hypotheses have been put forth:

1. The "*de novo*/maternal-precursor hypothesis" attempts to explain the observation that, due to a dramatic centrosome reduction, rodent sperm lack a recognizable centriole, yet centrioles are observed when the embryo reaches the 64-cell stage (Schatten *et al.* 1986; Gueth-Hallonet *et al.* 1993). It has been proposed that the embryonic centriole forms *de novo* (Howe and Fitzharris 2013). Alternatively, it has been claimed that the oocyte contains centriolar precursors that give rise to the embryo's centrioles (Calarco 2000). This hypothesis may also apply to some insects that reproduce by parthenogenesis (Ferree *et al.* 2006).
2. The "regeneration hypothesis" proposes that nonrodent mammalian sperm cells have an intact centriole (the proximal centriole) and, due to centrosome reduction, a degenerated centriole (the distal centriole). After fertilization, the degenerated centriole regenerates to form a second centriolar structure (Manandhar *et al.* 2005; Schatten and Sun 2009). It is claimed that both the intact and the "regenerated" centriole duplicate to form two pairs of centrioles, together resulting in three centrioles and one regenerated centriole.
3. The "duplication hypothesis" postulates that a single functional centriole is inherited from the sperm, which is duplicated soon after fertilization. In *Drosophila*, only one functional centriole is known (Fuller 1993); the second zygotic centriole is presumed to be the product of the sperm centriole duplicating in the zygote (Callaini and Riparbelli 1996). In this hypothesis, a round of centriole duplication happens prior to the zygote's first cell division.
4. The "paternal precursor hypothesis" theorizes that a sperm provides both a centriole and a centriolar precursor that originated during spermiogenesis, but did not mature to a centriole; in the zygote, the precursor becomes the second centriole (Crozet *et al.* 2000).

We have recently discovered that, in addition to the giant centriole (GC), which is attached to the plasma membrane and is equivalent to a basal body, *Drosophila* sperm contain an unidentified centriolar structure that lacks the distinctive structural characteristic of a centriole; we named it the proximal centriole-like (PCL) (Blachon *et al.* 2009). The PCL is a centriole precursor. Initially, the PCL forms in a similar way to a centriole, as they both use the same molecular pathways. However, the PCL diverges from the centriolar formation pathway prior to when a centriole acquires centriolar microtubules, a defining characteristic of a centriole. The PCL may be the predicted precursor of the precursor hypothesis.

Here we report that, like the GC, the PCL also undergoes centrosome reduction. As a result, currently, there is no way to stably label the PCL of the *Drosophila* sperm to directly test the PCL hypothesis. However, the PCL hypothesis can be differentiated from the duplication hypothesis and the maternal-precursor/*de novo* hypothesis using three criteria. First, the PCL hypothesis predicts that the first two zygotic centriolar structures appear in the zygote simultaneously, immediately after fertilization. Second, in the zygote that is generated from an oocyte expressing GFP-tagged early centriolar proteins, only the daughter centrioles of the first two zygotic centrioles should be labeled by GFP proteins that initiate centriole formation. Third, in a zygote that is generated from a mutant oocyte that cannot support centriole duplication, two centrioles should be observed after fertilization. Here, we test these predictions and provide evidence for the PCL/paternal precursor hypothesis.

Materials and Methods

Fluorescence microscopy

For embryo imaging, 50 male and 50 virgin female flies, <5 days old, were placed in an egg collection chamber with a grape agar plate with yeast paste. Chambers were used for 3 days and embryos were collected every 4 min. Immediately after collection, the embryos were placed in a mactech dish and washed with 100 μl of distilled water and then a wash buffer (0.7% NaCl + 0.05% Triton 100×). Afterward, 50% bleach solution was added onto the embryos until the appendages of the embryos disassociated. The embryos were rinsed twice with wash buffer, fixed in a 1:1 solution of heptane and methanol, and shaken vigorously by a vortex until the embryos settled down in the methanol layer. This was followed by removal of the fixative and suspending the embryos in acetone. At this stage, the embryos were usually stored at −20°. Then the embryos were rehydrated sequentially in 70, 50, 30, and 10% methanol in PBS and then in PBS alone. The embryos were then incubated in PBT (PBS+1% Triton) for 30 min and blocked in PBST (PBT+3% BSA) for 1 hr. Then the embryos were incubated with primary antibodies in PBST for 1 hr at room temperature. After three 5-min washes in PBT, the embryos were incubated with secondary antibodies and 1 μg/ml of DAPI for 1 hr at room temperature. The embryos were washed three times with PBT for 5 min each and then washed in PBS for 5 min. The embryos were mounted on a slide using a mounting medium (PBS, 50% glycerol, 0.5% N-propyl-gallate) and imaged. Testis imaging was performed as described in Basiri *et al.* (2013). Images were taken by a Leica SP5 or SP8 scanning confocal microscope as Z stacks. Maximal projection images were then modified using Adobe Photoshop and annotated using Adobe Illustrator.

Embryo development

To analyze the embryo development, 50 male and 50 female flies were allowed to mate overnight in a chamber. Then the agar plate with the laid embryos was incubated at 25° for 24 hr, and the number of larvae were counted.
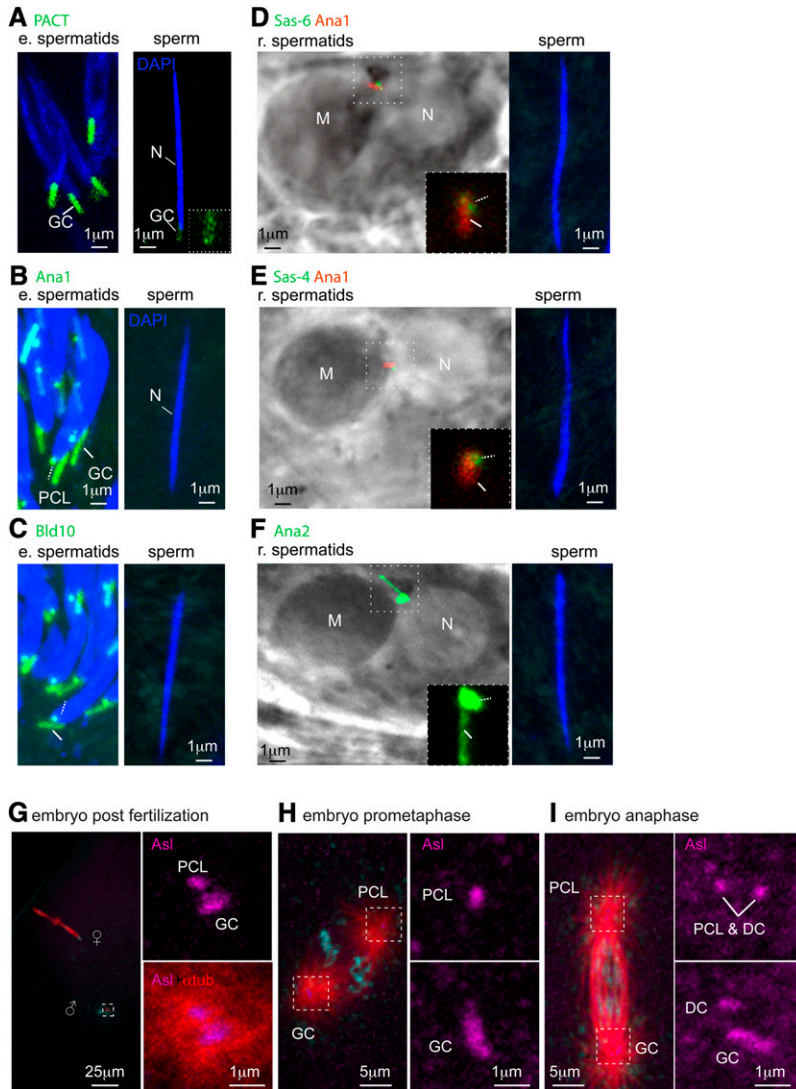
Figure 1 The PCL and GC undergo centrosome reduction and recruit PCM after fertilization. (A) The GC (solid line) is intensely labeled by PACT-GFP (which is overexpressed by the strong ubiquitin promoter in intermediate spermatids), but can be barely observed at the base of the sperm nucleus (see inset for magnification of this GC). (B–F) The PCL (dashed line) and GC (solid line) are observed in intermediate spermatids by Ana1-GFP (B) and BLD10-GFP (C), as are round spermatids by Sas-6 GFP (D), Sas-4-GFP (E), and Ana2-GFP (F). The GC is also marked by Ana1-td tomato (D–F). However, none of these proteins are observed in sperm found in the seminal vesicle. Note that Ana2-GFP (Stevens *et al.* 2010) is strongly expressed using the ubiquitin promoter, yet it is completely eliminated during centrosome reduction. N, nuclei. (G–I) *Drosophila* zygotes contain two centrioles immediately after fertilization and four during mitosis (G). After fertilization, and during maternal meiosis II (twin meiosis II spindles are labeled red, ♀), the GC and second centriolar structure, which is likely to be the PCL, are labeled by Asl; these structures form microtubule asters (red) that associate with the male pronucleus (♂). (H). During prometaphase, the GC and the PCL form distinct microtubule asters (I). During anaphase, the GC and the PCL are each accompanied by an Asl-labeled daughter centriole, indicating that they each gave rise to a DC.

Statistical methods

Experiments were repeated at least three times, and statistical analyses ($\pm$SEM) were done with GraphPad Prism 5. A two-tailed, unpaired Student's *t*-test was used.

Results

PCL and GC undergo centrosome reduction during late spermiogenesis

At the end of spermiogenesis, mammalian centrosomes undergo a process by which they lose their PCM and centriole structure is degraded, resulting in sperm with modified centrioles (Schatten 1994; Manandhar and Schatten 2000; Manandhar *et al.* 2000, 2005). Similarly, during *Drosophila* spermiogenesis, the PCM proteins γ-tubulin (Wilson *et al.* 1997) and Cnn (Li *et al.* 1998) are eliminated from the GC, indicating that centrosome reduction also happens in *Drosophila*. The GC marker PACT-GFP, which can be intensely observed in the spermatid GC, is hardly observed in the sperm GC (Figure 1A) (Martinez-Campos *et al.* 2004). To test if the PCL also undergoes centrosome reduction, and whether during centrosome reduction centriolar proteins are also eliminated from the GC, we studied the localization of GFP-tagged centriolar proteins during spermiogenesis and in sperm. We observed PACT-GFP, Ana1-GFP, BLD10-GFP, Ana2-GFP, Sas-6-GFP, and Sas-4-GFP in intermediate or round spermatids, in the giant centriole, and in the PCL (Figure 1, A-F). We found that all of these centriolar proteins are missing from mature sperm, indicating centrosome reduction takes place in both the GC and the PCL. This indicates that the PCL, which was formed during early spermiogenesis and without microtubules (Blachon *et al.* 2009), loses many of the proteins that formed it during centrosome reduction.

Zygote has two centrioles immediately after fertilization

After fertilization, the centrioles that lost their proteins via centrosome reduction can be detected by labeling the PCM components that they recruit from the oocyte's cytoplasm
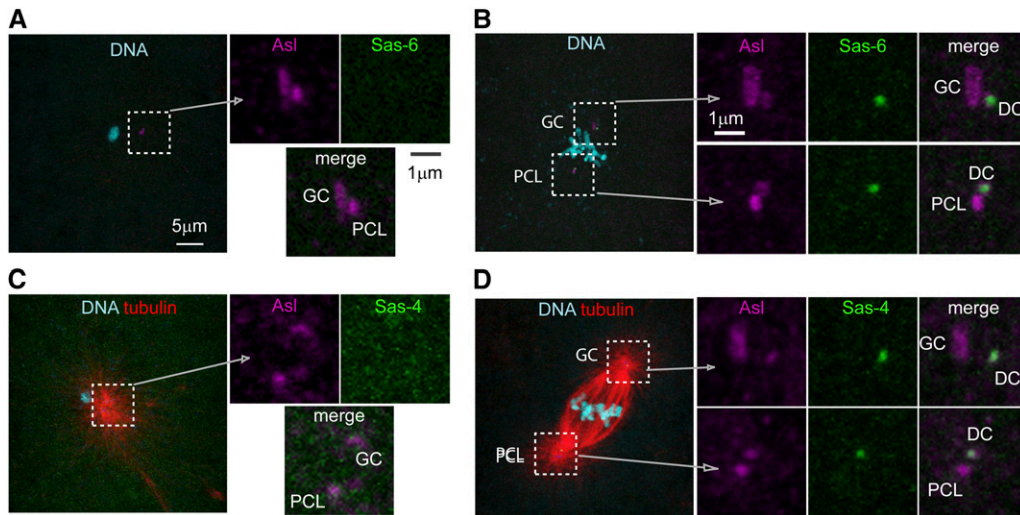
Figure 2 Maternally GFP-tagged centriolar proteins are incorporated into the daughter centrioles of the GC and the PCL. (A and C) Immediately post-fertilization, the first two zygotic centrioles are not labeled by maternal centriolar proteins, but are labeled by Asl (magenta). (B and D) During the zygote's first mitosis, an antibody against Asl labels all centrioles, but Sas-6-GFP (B) or Sas-4-GFP (D) labels only two centrioles. This indicates that three types of centrioles are observed: a GC, the presumed PCL, and two DCs.

(Callaini and Riparbelli 1996). To image the zygote's centrioles, we performed immunofluorescence using an antibody against the PCM protein Asterless (Asl) and an anti-α-tubulin antibody that labels microtubule asters (Blachon *et al.* 2008). We found that immediately after fertilization, when the twin meiosis II spindles of the female are observed, the two Asl-labeled centrioles are present in the zygote. One of the centrioles is longer and is likely to be the GC (Riparbelli and Callaini 2010), and the other one is a smaller centriole (Figure 1G). Because the two centrioles are observed immediately post-fertilization, this second smaller centriole is likely to be derived from the PCL (Figure 1G, left; see below). Both centrioles are marked by Asl, which they must have recruited from the cytoplasm, together with the rest of the PCM, to form their own aster microtubules. This suggests that both the GC and PCL, which lose many of their components during centrosome reduction, are capable of recruiting PCM and anchoring astral microtubules. During the zygote prometaphase, the two centrioles are at opposite spindle poles (Figure 1H). During anaphase, centriole duplication has already occurred, and daughter centrioles are present near each of the pre-existing centrioles [daughter centrioles (DC), Figure 1I]. These data suggest that the PCL, like a typical centriole, can serve as a scaffold for daughter centriole formation and forms a second centriole. Altogether, these data indicate that either the sperm transported both the GC and the PCL or that the GC duplicated immediately after fertilization and before the completion of maternal meiosis II.

Unlike their daughter centrioles, the first two zygotic centrioles do not incorporate centriolar proteins

Centrioles, during their formation, incorporate centriolar proteins such as Sas-6 and Sas-4, which are available in the cytoplasm (Kirkham *et al.* 2003; Leidel and Gonczy 2003). Therefore, to test whether the first two zygotic centrioles are formed in the oocyte, we used oocytes expressing the centriolar proteins Sas-6-GFP and Sas-4-GFP. These oocytes

were fertilized with sperm centrioles, which can be observed once they recruit PCM. As expected, immediately after fertilization, there are two zygotic centrioles labeled by the PCM protein Asterless (Figure 2, A and C). However, neither of these centrioles was labeled by Sas-6-GFP or Sas-4-GFP, indicating that they did not form in the zygote and that their origin must be from the sperm. One of these centriolar structures is large, suggesting that it is the GC, while the second is smaller, suggesting that it is the PCL. Later, during mitosis, Asterless staining identifies four centrioles. Of these, two are labeled only by Asterless, and the remaining two are labeled by Asl and Sas-6-GFP or Sas-4-GFP (Figure 2, B and D). The two centrioles labeled by only Asterless are likely to be the GC and the PCL. The two centriolar structures labeled by Sas-6-GFP or Sas-4 -GFP are likely to be the DCs of the GC and the PCL (Figure 2, B and C, left).

Altogether, this experiment strongly argues against both the maternal precursor hypothesis and the duplication hypothesis. Indeed, maternal centrioles and centrioles duplicated in the zygote should have been labeled by Sas-6-GFP or Sas-4-GFP. These data, however, are consistent with the PCL hypothesis.

Homozygote *asl^mecD* zygotes have two centrioles

Drosophila Asterless and its vertebrate homolog Cep152 are essential proteins for centriole duplication (Blachon *et al.* 2008) and embryo development (Varmark *et al.* 2007). Depletion of Cep152 prevents both centriole duplication and Plk4-induced *de novo* centriole formation (Cizmecioglu *et al.* 2010; Hatch *et al.* 2010), indicating that Asterless/Cep152 is essential for centriole formation by both the duplication and *de novo* pathways. We recently showed that *asl^mecD* completely blocks centriole duplication in flies (Blachon *et al.* 2008). Because the *asl^mecD* flies die shortly after emerging from their pupal cases, these flies are not capable of mating. To remedy this, we generated *asl^mecD* oocyte clones that lack the Asterless protein in heterozygous *asl^mecD* females (Supporting Information, Figure S1, File S1). To test for the role of Asl in
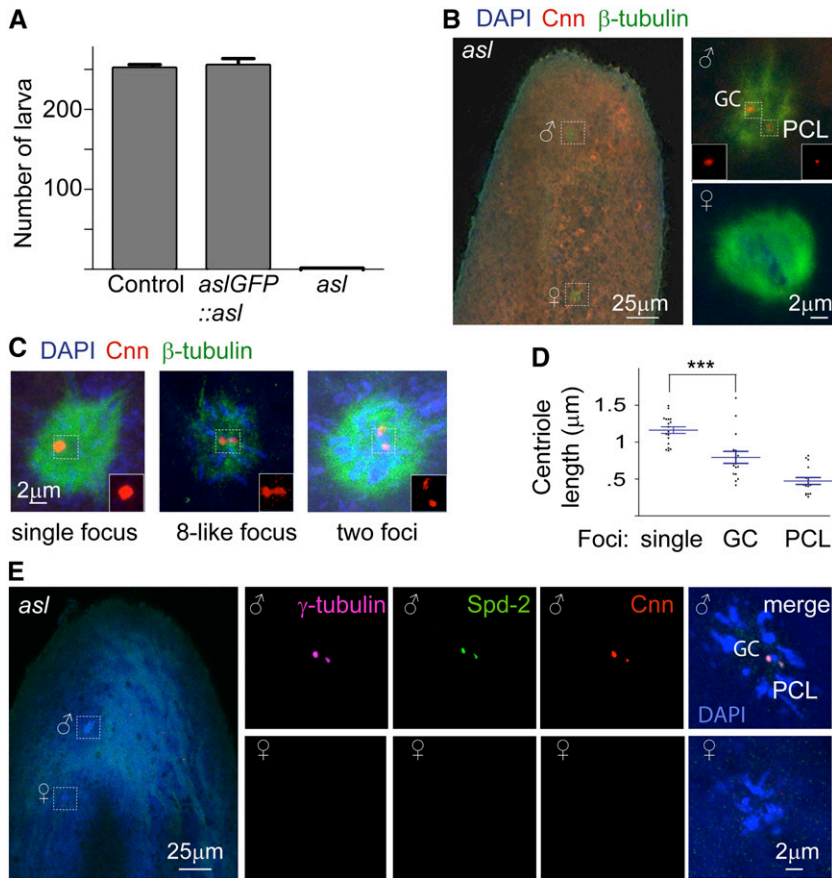
Figure 3 Two centrioles are observed in an oocyte mutant for centriole duplication. (A) Embryo development is arrested in embryos generated by asl^{mecD} oocytes and is rescued in the presence of Asl-GFP. (B) A zygote generated by an asl^{mecD} oocyte with a paternal pronucleus (♂) labeled by two Cnn-labeled centrioles and a maternal pronucleus (♀) lacking centrioles. (C) Paternal pronuclei in zygotes generated by asl^{mecD} oocytes have two centrioles that are superimposed (single focus), near each other (8-like focus), or apart from each other (two foci). (D) Graph depicting the length of the Cnn foci in embryos with one Cnn focus (single) as well as long and short CNN foci in embryos with two separate Cnn foci, demonstrating that most of the apparent foci are two superimposed foci. ***$P < 0.0003$. (E) Cnn, γ-tubulin, and Spd-2 are colabeling the two zygotic centrioles.

fertilization, these adult females were mated to normal males, and their zygotes were analyzed. As expected, we found that asl^{mecD} embryos cannot develop into larvae (Figure 3A). Importantly, introduction of Asl-GFP using germline transformation reverted the asl^{mecD} phenotype, allowing embryo development (Figure 3A).

We then analyzed the phenotype of zygotes generated by asl^{mecD} oocytes. Embryos collected immediately after they were laid were stained with the nuclear marker DAPI, an antibody for microtubules, and an antibody for the centrosomal protein Cnn. Two populations of embryos were found. The first population has two to four nuclei associated with the microtubule spindles, which are lacking centrosomes as judged by the absence of PCM markers (36% or 28/77), indicating that they are unfertilized oocytes. The second population has one paternal pronucleus that possesses Cnn labeling and one maternal pronucleus that lacks Cnn labeling, indicating that the second population are fertilized oocytes (*i.e.*, an embryo) (64% 49/77) (Figure 3B). The maternal and paternal pronuclei were not close to each other, indicating that the embryo's development arrested before completion of pronuclei migration.

Analysis of the Cnn labeling of the paternal pronuclei found that 32% of embryos (16/49) have two clearly separated Cnn foci; 30% (15/49) have two close, but distinct, Cnn foci that look like the "figure 8"; and 36% (18/49) have a single Cnn focus (Figure 3C). Because the

size of the single Cnn focus is larger than a clearly separated Cnn focus (Figure 3D), it is likely that many of the single foci are two centriolar structures that are closely associated with each other. Similar to Cnn, γ-tubulin and DSpd-2 also label both centrioles of the zygote generated by the asl^{mecD} oocytes (Figure 3E). Altogether, this analysis demonstrates that an embryo that cannot duplicate centrioles has two centriolar structures, consistent with the PCL hypothesis.

Discussion

We have shown previously that the *Drosophila* sperm contain a second centriolar structure, the PCL (Blachon *et al.* 2009). In this article, we demonstrated that two centrioles are present in the zygote after fertilization; that these two centrioles are not marked by maternally contributed centriolar proteins; and that, in a zygote unable to duplicate centrioles, there are two centrioles present. Therefore, the sperm must have brought two centrioles to the zygote.

We therefore propose the following model for centriole inheritance in *Drosophila*: the sperm provides a GC and a PCL, and both lack PCM and many centriolar proteins, while the oocyte is lacking any centrioles (Figure 4A). Immediately after fertilization, both the GC and the PCL recruit the PCM and nucleate astral microtubules, but they do not incorporate centriolar proteins (Figure 4B). Later, the centriole and PCL each template a daughter centriole, which is
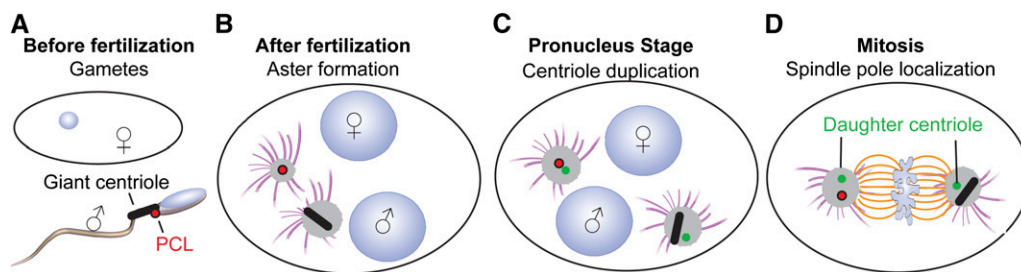
Figure 4 The PCL in *Drosophila* fertilization. (A) During fertilization, the oocyte lacks centrioles, whereas the sperm contains a GC and a PCL. (B) After fertilization, the centriole and the PCL form asters. (C) During the pronuclear stage, the centriole and PCL are duplicated, resulting in two DCs. (D) During mitosis, each pole has a centrosome that consists of a centriole with its daughter or of a PCL with its daughter.

made from maternally contributed proteins (Figure 4C). Finally, the two centrosomes, one derived from the GC, and the other derived from the PCL, migrate to the spindle poles of the embryo's first spindle (Figure 4D). Because after fertilization the PCL does not incorporate maternal Sas-6 and Sas-4, it is possible that the PCL does not mature to a typical centriole.

Our data extend the similarity between the PCL and centrioles. First, like the GC, the PCL also undergoes centrosome reduction at the end of spermiogenesis. Second, after fertilization, both the GC and the PCL do not recruit centriolar proteins, despite losing them. The GC and the PCL still recruit PCM proteins, form astral microtubules, and give rise to a daughter centriole. How the GC and the PCL can accomplish this without Sas-6 and Sas-4, and possibly other centriolar proteins, remains a mystery.

Importantly, the PCL hypothesis may be applicable to vertebrates; it may explain why only three centrioles have been observed in a mammalian zygote during mitosis (Sathananthan *et al.* 1996; Crozet *et al.* 2000), although it is known that four centrioles are required for normal mitosis in a cell. Perhaps the PCL, which was unseen in any organism until our discovery in *Drosophila*, remains undiscovered in mammalian sperm.

The finding that the PCL is the second centriole that the sperm provides argues for an almost universal mechanism of centriole inheritance among animals that involves paternal inheritance of two centrioles. These may be two centrioles (*C. elegans*, frogs and sea urchin), a regenerated centriole (or potentially a PCL), and one centriole (nonrodent mammals) or one centriole and one PCL (*Drosophila*). Altogether, we have shown here that the PCL is a centriole precursor that contributes to the first zygote in *Drosophila* and, as a result, is the origin of half of the fly's centrioles. Similar mechanisms may exist in other animals.

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GENETICS

The Origin of the Second Centriole in the Zygote of *Drosophila melanogaster*

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♂   ♀

Bloomington stocke 2149
w;; P{neoFRT}82B P{ovoD1-18,W}3R / TM3, Sb   **X**   Bloomington stocke **7**
P{hsFLP}1, y w;; Dr[Mio] / TM3, Sb

(**F1**) P{hsFLP}1, y w;; P{neoFRT}82B P{ovoD1-18}3R / TM3, Sb **X**   w; ;P{neoFRT}82B Asl-mecD / Tm6B

heat shock

w   **X**   P{hsFLP}1, y w/w;; P{neoFRT}82B P{ovoD1-18}3R (**F2**)
/ P{neoFRT}82B Asl-mecD

Embryo collection

Figure S1   Generating an embryo from *asl^mecD* homozygote oocytes (related to Figure 4). We recently showed that *asl^mecD*, unlike any other *Drosophila* mutant, completely blocks centriole duplication (Blachon *et al.* 2008). Since the *asl^mecD* flies die shortly after emerging from pupa, these flies are not suitable for mating. This lethality can by avoided by using heterozygous *asl^mecD* females. However, these females provide wild type *asl* mRNA to their oocytes via the maternal contribution; thus, centriole duplication will not be completely blocked in these oocytes. To address this, we generated oocytes that completely lack the Asl protein except in heterozygous *asl^mecD* females. For this, we created germline *asl* mutant fly lines that lacked any *asl* maternal contribution. This was done using a FLP-FRT recombination technique and *ovo^D1* mutation that blocks the formation of oocytes that are not *asl^mecD* homozygotes (Chou and Perrimon 1996). Recombination was induced in larvae via a heat shock-inducible Flippase enzyme. Thus, as adults, they produced homozygous *asl^mecD* oocytes that lacked any Asl protein. Adult females were then mated to wild type males and their zygotes were analyzed.

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Transgenic Flies

All *Drosophila* stocks were cultured on standard media at 25°C. Ana1-GFP, Ana1-tdtomato, Bld10-GFP, Asl-GFP, Sas-6-GFP, and Sas-4-GFP are expressed to near physiological levels using their corresponding promoter, and were previously described (BLACHON *et al.* 2009; BLACHON *et al.* 2008). Ana2-GFP is expressed under the strong ubiquitin promoter (STEVENS *et al.* 2010). Bloomington stock number 7 (P [hsFLP],y^1 w^{1118};;Dr^{Mio}/TM3,ry* Sb) and 2149 (w*;;P[neoFRT]82B P[ovoD$^{1-18}$,W$^+$]3R/TM3) were used to generate *asl^{mecD}* mutant embryos. Bloomington stock 5748 (P[neoFRT]82B cu^1 sr^1 e^s ca^1) and meiotic recombination was used to make the fly containing FRT82B and *asl^{mecD}*.It was selected for the presence of neomycin resistance and lack of *cu,* and was confirmed by failure to complement *asl^{mecD}*.

Antibodies

The following primary antibodies were used for immunofluorescence at the indicated concentrations: Guinea-pig anti-Cnn, 1:200 (a kind gift from Thomas C. Kaufman); mouse anti-β-tubulin,1:50 (Developmental Studies Hybridoma Bank); Rabbit Anti-Asl, 1:200 (Ap1193. (BLACHON *et al.* 2008)); mouse anti- -tubulin, 1:200 (Sigma); Rabbit anti-alpha-tubulin, rabbit anti-DSpd-2 1:200 (a kind gift from Maurizio Gatti); Rabbit anti-GFP (1:200; Fitzgerald Industries). The following secondary antibodies were used: Alexa Fluor® 488-conjugated goat anti-mouse IgG, 1:800; Alexa Fluor® 647-conjugated goat anti-guinea pig IgG, 1:800; Cyanine Cy3-conjugated goat anti-rabbit IgG,1:800 ; Rhodamine goat anti-mouse 1:200; Rhodamine goat anti-rabbit 1:200 (Jackson ImmunoResearch). DAPI was used at final concentration of 1 μg/ml (Sigma).

Generating homozygote *asl^{mecD}* embryos

Three crosses were performed. In the first cross, 50 males having the FRT (Flippase recognition target) were crossed with the 50 females having Flippase enzyme coding gene (FLP) on the first chromosome. The resultant F1 generation males were selected which had FLP gene on the first chromosome and FRT site on the third chromosome along with TM3 balancer. In the second cross, 50 males from the F1 generation were then crossed with 50 females, which were heterozygous for *asl^{mecD}* having a FRT site along with *asl^{mecD}* on the third chromosome. After 3 days, the flies were moved to a new vial and the vial with embryos was subjected to heat shock at 37°C for 1 hour. The heat shock was performed each day for the next 3 days, thus activating the Flippase enzyme and carrying out the recombination reaction at the FRT site and making the resultant F2 homozygous for *asl^{mecD}* mutation. The females lacking TM6B (*Humeral$^+$*) were selected from this F2 generation, and then were finally crossed with the wild type males and *asl^{mecD}* mutant embryos were collected.

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