Procentriole assembly without centriole disengagement: a paradox of male gametogenesis

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Summary
Disengagement of parent centrioles represents the licensing process to restrict centriole duplication exactly once during the cell cycle. However, we provide compelling evidence that this general rule is override in insect gametogenesis where distinct procentrioles are generated during prophase of the first meiosis when parent centrioles are still engaged. Moreover, the procentriole number increases during the following meiotic divisions and up to four procentrioles were found at the base of each mother centriole. However, procentrioles fail to organize a complete set of A-tubules, so being unable to work as template for centriole formation. Such a system, in which procentrioles form but halt growth, represents a unique model to analyze the process of cartwheel assembly and procentriole formation.

Running title: Procentriole assembly

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Introduction

The proper segregation of chromosomes during cell division into the two daughter cells relies on the correct organization of the bipolar spindle by just duplicated centrosomes. This is a crucial step, since abnormal centrosome numbers may lead to multipolar spindles with ensuing aneuploidy and chromosome fragmentation, forms of genomic instability that represent an established hallmark of cancer cells (Ganem et al., 2009; Crasta et al., 2012; Vitre and Cleveland, 2012). Since centrioles mark the sites where the centrosomal material is recruited their number determines the number of centrosomes (Sluder and Rieder, 1985) and, therefore, centriole duplication has to be accurately regulated. Although, vertebrate cells make temporal and spatial constraints to ensure that only one daughter is assembled for each mother once during the cell cycle, the mechanisms managing these controls are not fully understood. Centriole disengagement during metaphase/anaphase has been proposed to represent the licensing factor to allow centriole duplication during the following interphase (Tsou et al., 2009; Wang et al., 2011). Moreover, a newborn daughter centriole seems to be sufficient to inhibit the formation of additional sisters at the basal end of the mother by locally limiting the availability of structural and/or regulatory proteins needed for their assembly (Uetake et al., 2009; Brito et al., 2012).

Recent studies have suggested that a core module of specific conserved proteins drives the formation of daughter centrioles in the presence of pre-existing mothers or during de novo assembly (Gönczy, 2012). However, ultrastructural data fail to unambiguously reveal the same intermediate structures during the process of procentriole assembly in different organisms. It is still unclear if the structural details observed at the onset of procentriole formation may differ in various animal groups or if technical challenges may have prevented a careful analysis of the early steps of centriole assembly. Therefore, an increased understanding of centriole biogenesis in different animal systems is expected to shed light on the general mechanism of centriole duplication and assembly. We report here that distinct procentrioles appear at the base of duplicated centrioles during prophase of the first male meiosis in butterflies despite being engaged. Moreover, during the following meiotic divisions the procentrioles increase in number and form distinct clusters at the proximal end of each mother centriole. However, procentrioles fail to grow and are thus unable to organize centrosomal material.
Results and Discussion

Centriole engagement does not prevent procentriole assembly

As usual in insects, the butterfly primary spermatocytes had two centriole pairs disposed in V-shaped configurations that represent the platform for the assembly of four long “cilium-like” projections (Fig. 1a,b) (Henneguy, 1898; Meves, 1903; Friedlander and Wahrman, 1970; Wolf and Traut, 1987, Yamashiki and Kawamura, 1998). Unlike most animal cells in which the cilia are reabsorbed to release the centriole that will organize the spindle poles, the “cilium-like” projections persist during insect spermatogenesis to later organize the sperm axoneme (Riparbelli et al., 2012; Gottardo et al., 2013). Thus, the centriole performed the double role of spindle pole organizer and axoneme constructor. While studying the dynamic of the “cilium-like” projections during spermatogenesis and spermatid differentiation in the butterfly Pieris brassicae we found distinct structures close to the basal region of each centriole (Fig. 1c,d). These structures appeared as short cylinders of nearly constant size with an average diameter of 114.2 nm (±4.1; n= 14) and an overall length of 117.2 nm (± 3.7; n=9), longitudinally crossed by an inner tubule of about 21.3 nm (±2.6; n= 14) in diameter. The tubule is linked to the wall of the cylinder by thin radial projections (Fig. 1e). Such architecture is reminiscent of the cartwheel found in procentrioles, the early intermediates in centriole assembly of most animal cells. Procentrioles were lacking in young primary prophase spermatocytes, but they were always found during late prophase. Thus, the primary spermatocytes showed four fully elongated centrioles along with four procentrioles. This suggests that the centrioles of primary prophase spermatocytes underwent an extra duplication cycle, in addition to the usual duplication that occurs in early prophase soon after the last spermatogonial mitosis. This represents an unconventional condition, since in animal cells the centrioles duplicate once and only once in every cell cycle during transition from G1 to S of interphase (Wong and Stearns, 2003) to avoid the formation of multipolar spindles. It is generally believed that breaking of the close association, or disengagement, between mother and daughter centrioles during metaphase/anaphase transition represents the licensing factor for the forthcoming duplication during interphase of the next cell cycle (Tsou and Stearns, 2006; Tsou et al., 2009). Accordingly, ultrastructural analysis of Drosophila primary spermatocytes (Tates, 1971; Fritzi-Nigli and Suda, 1972; Riparbelli et al., 2012), in which centrioles are linked together by fibrous material (Stevens et al., 2010), failed to reveal procentrioles. In butterfly primary spermatocytes, the centrioles within each pair were held together by thin fibrous material, like in Drosophila centrioles, but they duplicate. This represents a remarkable exception in the regulation of centriole duplication during the cell cycle and raises questions on the universality of the licensing factor.
The radial spokes connected the central hub to the wall of the procentriole (Fig. 1e) that was formed by a thin peripheral ring surrounded by a cluster of dense material. The ring was more evident at the distal end of the procentriole where the dense material was scarce (Fig. 1f). Short microtubules contacted the outer side of the procentriole wall that was also crossed by curved grooves whose diameter was similar to that of single microtubules (Fig. 1e,g). Some of the grooves underwent partial closure suggesting that they may represent intermediate stages in microtubule assembly. The outer dense wall of the procentriole may be regarded, therefore, as the platform for the assembly of microtubules and the curved grooves may denote nascent A-tubules. Strikingly, electron-dense hook structures, of unknown composition, were observed on the tube of Caenorhabditis prior to the presence of assembled microtubules (Dutcher, 2007). The A-tubule formation in butterflies would require a process of lateral nucleation, such that described for B- and C-tubules assembly during centriole formation in human cultured cells (Guichard et al., 2010). In cultured cells, however, the B-tubule utilizes as template the A-tubule that is presumably nucleated by a proximal cap of \( \gamma \)-tubulin.

**A model of procentriole assembly**

The parent centriole was surrounded by two concentric sheets of dense material: a complete inner and an incomplete outer (Fig. 1h). The hub extended beyond the procentriole length and reached the inner dense sheath that surrounded the base of the parent centriole (Fig. 1i,j). Radial projections cannot be distinguished in the proximal region of the hub. Likewise, a distinct stalk connects the central hub to the parent centriole in human cells (Guichard et al., 2010). These observations suggest that the hub may emerge from the dense sheet at the base of the mother centriole to initiate the organization of a central structural scaffold on which radial spokes assemble to originate the primitive cartwheel. A thin cylinder may be then built around the radial spokes (Fig. 1k). Therefore, the A-tubules do not attach to the distal end of the radial spokes by distinct pinheads, as reported in other systems (Guichard et al., 2013), but contact a thin cylinder that is the scaffold on which the material presumably involved in the nucleation of the A-tubule will be recruited.

Our observations are consistent with a model in which a small tube may represent a first step in procentriole assembly. Consistently, the over-expression of Sas-6 in Drosophila led to the appearance of tube-like structures (Rodrigues-Martins et al., 2007). Moreover, the concurrent over-expression of Sas-6 with its binding partner Ana2 led to the formation of cartwheel-like structures in Drosophila spermatocytes (Stevens et al., 2010). The assembly of a large tube that forms near the parent centriole characterizes the first step of procentriole formation in Caenorhabditis (Pelletier et al., 2006).
Daughter centriole assembly does not require continuity with its mother

Each parent centriole has its procentriole during the first meiotic prophase (Fig. 1c,d). When the cilium-like projections elongated further during prometaphase (Fig. 2a) two procentrioles were seen in the proximity of each parent centriole (Fig. 2b,c; n=7). The spindle poles of secondary spermatocytes during metaphase/anaphase contained only one centriole (Fig. 2d) at the base of the cilium-like projections (Fig. 2e), but a cluster of two (Fig. 2f,g; n=9), three (Fig. 2h,i; n=5), and sometimes four (n=2) procentrioles was found near the basal region of each parent centriole. Therefore, multiple daughter procentrioles can be concurrently generated at the base of single parent centrioles challenging the traditional view of a preferential site of assembly at the base of each parent and its role in managing procentriole formation (discussed in Sluder and Khodjakov, 2010).

Besides the temporal control of the once and only once formation during the cell cycle, animal cells also have a spatial control to yield the formation of only one procentriole at each existing mother despite the large pool of cytoplasmic components available (Nigg and Stearns, 2011). Once the assembly of the new procentrioles has been initiated, further centriole duplication is inhibited until the cells pass through mitosis (Wong and Stearns, 2003; La Terra et al., 2005; Uetake et al., 2007). It is possible that the numerical control of procentriole formation is based on a balanced control among protein-protein interactions and/or activations. Therefore, procentriole formation would be restricted to the vicinity of the existing centrioles that maintain a compact region of pericentriolar material, the only area in the cell where new centrioles can form (Loncarek et al., 2008, 2010). Antibodies against DSas-6, Ana1 and Bld10/Cep135 that recognize a procentriole-like structure (PCL) at the base of the mother centriole in *Drosophila* elongating spermatids (Blachon et al., 2009, 2014; Mottier-Pavie and Megraw, 2009) did not cross-react with butterfly centriolar proteins. By contrast, an antibody against Sak/Plk4 that plays in *Drosophila* main roles in the assembly of the PCL (Blachon et al., 2009) recognised in *Pieris* additional spots for each centriole (Fig. 2j).

The concurrent appearance of multiple centrioles has been described under experimental conditions, in which the concentration of proteins involved in centriole biogenesis or cell-cycle regulation has been modified (Brownlee and Rogers, 2012). Clusters of procentrioles in flower-like or rosette disposition are, indeed, observed around each mother after the overexpression of Plk4 kinase (Habedanck et al., 2005; Kleylein-Sohn et al., 2007), a process enhanced by Cyclin-E/CDK2 (Duensing et al., 2007). A similar phenotype is also induced by down-regulation of the ubiquitin ligase SCF<sub>Slimb</sub> complex that regulates Plk4 levels (Cunha-Ferreira et al., 2009; Rogers et al., 2009). The rosette phenotype is also generated by the overexpression of Sas-6 (Strnad et al., 2007), a
protein needed for cartwheel assembly, or by the overexpression of STIL, the putative partner of Sas-6 (Tang et al., 2011; Arquint et al., 2012.). More than one daughter centriole at single mother also forms following proteasome inhibition or HPV-16 E7 oncoprotein transfection (Duensing et al., 2007). In addition, some cultured vertebrate cells assemble multiple daughter centrioles around each mother, when blocked in S-phase (Balczon et al., 1995). Multiple basal bodies also arise from a single mother in a flower-like disposition in mammalian differentiated epithelial cells such as the ones of the respiratory and reproductive tracts (Anderson and Brenner, 1971; Dirksen, 1971). However, under normal circumstances only one daughter forms in cycling cells. Therefore, the finding of multiple procentrioles at each spindle pole in butterflies seems to create a paradox and suggests that the centriole duplication control is down regulated. The rule of “only one centriole forming per mother” may overcome, suggesting that the assembly of a daughter does not necessarily prevent the formation of additional ones.

Procentrioles appear in butterfly during late primary prophase, when the parent centrioles attained their full size and do not elongate further. Moreover, they did not undergo significant shape changes and a complete set of A-tubule never forms. This suggests that centriole elongation is correlated to the cell cycle with full elongation taking place at the end of the first meiotic prophase. The failure of procentriole growth and maturation in butterfly spermatocytes may represent the limiting factor to avoid the assembly of functional centrosomes and the formation of supernumerary spindles. Therefore, the assembly of additional centrioles is blocked not at the beginning, but at the tubule addition stage. *Pieris brassicae* spermatids lack a distinct centriole adjunct. Thus, it could be easier to detect procentrioles close to basal bodies. One or two procentrioles were observed in early spermatids (Fig. 3a,b). However, they lost the close association with parent centrioles that nucleated the spermatid axoneme (Fig. 3c) and were often found next to the nuclear membrane (Fig. 3d). Procentrioles found in early spermatids had similar diameter than those found during preceding meiotic divisions (112.4 nm (±3.2; n=5) vs. 114.2 nm (±4.1; n= 14), respectively) and an invariable cartwheel structure, but were slightly shorter (81.7 nm (±2.9; n=6) vs. 117.2 nm (±3.7; n=9). Procentrioles were not longer detected in elongating spermatids. These results are consistent with the gradual reduction of the supernumerary procentrioles and point to a redundant role of these structures during butterfly gametogenesis.

**A procentriole at the core of the parent centriole**

Parent centrioles at the end of the first prophase had a nearly constant size with an average diameter of 195 nm and an overall length of 730 nm (Fig. 4a). The proximal end of the mature centriole showed a dense ring connected to the central hub by nine radial spokes (Fig. 4b). This structure that
we termed here the “inner ring complex”, i.e. IRC, can be recognized in longitudinal sections as a short cylinder within the basal region of the centriole (Fig. 4a). Microtubule triplets appeared in slightly high sections. Triplets were outline by a second outer ring of dense material (Fig. 4c). The IRC disappeared in further distal sections, whereas the outer ring became undulated (Fig. 4d) and was barely detectable in the distal region of the centriole (Fig. 4e). The hub increased diameter moving from the basal end of the centriole (Fig. 4b,c,d) and disappeared concurrently with the peripheral ring. Sections in which we had the opportunity to simultaneously find the basal end of the parent centrioles and associated procentrioles (Fig. 4f,g,h) showed that the overall fitting of these structures is excellent. Thus, the procentrioles may correspond to distinct platforms, on which the future centrioles will be assembled. A cartwheel is transiently present at the base of young centrioles in vertebrate centrosomes, but disappears in mature centrioles (Alvey, 1986; Strnad and Gönczy, 2008; Azimzadeh and Marshall, 2010).
Materials and Methods

Insects
A culture of *Pieris brassicae* was obtained from a laboratory strain and reared on ventilated cages at 20°C. Larvae were fed with cabbage leaves until pupation.

Antibodies
We used the following antibodies: rabbit anti Sak-Plk4 (1:200; Bettencourt-Dias et al., 2005), chicken anti-DSAS-6 (1:1000, Rodriguez-Martins et al., 2007), rabbit anti-Bld10 (1:500, Mottier-Pavie and Megraw, 2009), mouse anti-acetylated tubulin (1:100; Sigma-Aldrich). The secondary antibodies used (1:800, InVitrogen) were conjugated with Alexa 488 or Alexa 555.

Immunofluorescence preparations
For immunostaining testes from pupae of the lepidopteran *Pieris brassicae* were dissected and fixed according to Riparbelli e al. (2012). For localization of axonemal microtubules and centriolar proteins the samples were processed as described previously (Riparbelli et al. 2013). DNA was visualized with Hoechst 33258. Images were taken with an EC Plan-Neofluar 100x/1.30 objective by using an AxioImager Z1 (Carl Zeiss) microscope equipped with an AxioCam HR camera (Carl Zeiss).

Transmission electron microscopy
Testes isolated from pupae were fixed in 2.5% glutaraldehyde in PBS overnight at 4°C and further processed as described previously (Gottardo et al., 2013). Thin sections were stained routinely with uranyl acetate and lead citrate, and then observed with a Tecnai Spirit EM (FEI) equipped with a Morada CCD camera (Olympus).

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Author contribution
M.G., M.G.R. and G.C. conceived the project. M.G. and M.G.R. performed experiments. G.C. wrote the manuscript.
References


Figure legends

**Fig. 1. Procentriole in primary spermatocytes.** (a) Primary butterfly spermatocytes have four elongated cilium-like projections (green); DNA is red. (b) The axoneme of the cilium-like projections is nucleated by elongated centrioles. (c,d) One procentriole (arrow) is found near the proximal region of parent centrioles. (e,f) Consecutive cross sections through the proximal and distal ends of the procentriole shown in c: a single microtubule is visible on the procentriole wall (asterisk); a thin inner ring (double arrow) is more apparent where the dense material surrounding the procentriole is scarce. (g) Curved grooves are visible on the wall of the procentriole (asterisks). (h) The parent centriole is surrounded by inner (double arrowhead) and outer (arrow) sheets of dense material; the proximal end of the procentriole leans against the outer sheet, whereas its distal end contacts a flat cistern. (i,j) The hub (arrowheads) reaches the inner sheet that surrounds the basal end of the mother centriole. (k) Possible steps in butterfly procentriole assembly. Scale bar = 2.5 μm in a, 500 nm in b, 200 nm in c-d, 50 nm in e-j.

**Fig. 2. Multiple procentrioles form at the base of single mothers.** Prometaphase of the first meiosis: (a) Cilium-like projections are very elongated; microtubules are green, DNA is red. (b) Details of centriole and (d) procentrioles at one spindle poles. Metaphase of the second meiosis: The spindle poles contain only one centriole (d) that nucleates a cilium-like structure (e; microtubules are green, DNA is red). Details of centrioles (f,h) and procentrioles (g,i) visible in associated serial sections during metaphase/anaphase of the second meiosis. (j) Localization of Sak-Plk4 cross-reacting antigens (red) at the base of the cilium-like projections (green) during metaphase of the second meiosis: two small spots are visible (arrow). Scale bar = 2.5 μm in a and e; 200 nm in b, f and h; 500 nm in d; 50 nm in c, g and i; 1.5 μm in j.

**Fig. 3. Procentrioles persist in young spermatids.** (a) Early spermatids have a round nucleus (red, DNA) and an elongate axoneme (green, microtubules). (b) The centriole that nucleates the spermatid axoneme contacts the nucleus. (c,d) Procentrioles are often close to the nuclear envelope (arrow). Scale bar = 2.5 μm in a, 200 nm in b, 100 nm in c and d.

**Fig. 4. Procentrioles within the parent centrioles.** (a) The basal region of the centriole contains a short cylinder (arrowheads). (b-e) Consecutive cross sections through a parent centriole: the basal end shows a thick ring (arrowheads) on which microtubule triplets assemble. The central hub increases diameter moving far from the proximal end of the centriole (small arrowheads). The ring
disappears in further sections. (f-h) Longitudinal and cross sections of two orthogonal centrioles: note the overlapping of the basal end of the parent centriole (arrows) and the procentriole (arrowhead). Scale bar = 100 nm.