Procentriole elongation and recruitment of pericentriolar material are downregulated in cyst cells as they enter quiescence

Maria Giovanna Riparbelli, Gabriele Colozza and Giuliano Callaini*

University of Siena, Department of Evolutionary Biology, Via A.Moro 2, I-53100 Siena, Italy
*Author for correspondence (callaini@unisi.it)

Summary

The apical region of the *Drosophila* testis contains a niche with two stem cell populations: germline stem cells (GSCs) and cyst progenitor cells (CPCs). Asymmetrical division of these stem cells leads to gonioblast daughters (which undergo further mitoses) and cyst cell daughters (which withdraw from the cell cycle and become quiescent). Although a considerable body of evidence indicates important roles for centrosomes in spindle orientation and asymmetrical division of GSCs, the behaviour and function of the centrioles in CPCs and their daughters remain unknown. Here, we show that quiescent cyst cells lose centrosome components after two divisions of the spermatogonia they envelop, but keep the centriolar component SAS-6. Cyst cells do have centriole pairs, but they are formed by a mother and a very short daughter that does not elongate or mature. The presence of procentrioles in quiescent cyst cells suggests that the centriole duplication cycle is uncoupled from the G1-S transition and that it might begin even earlier, in mitosis. Failure to enter the cell cycle might result in the improper recruitment of centriolar components at the mother centriole, thus hampering the full elongation of its daughter. Procentriole maturation defects could thus lead to the inability to maintain centrosomal components during development.

Key words: *Drosophila* spermatogenesis, Stem cell niche, Cyst cells, Centrosomes, Centrioles

Introduction

Stem cells have a key role in generating hundreds of specialized cell types during embryonic development and in replacing short-lived differentiated cells in adult tissues. To achieve this task, stem cells have the potential to generate identical copies of themselves, whilst also producing daughter cells that are committed to differentiate. This ability is tightly related to unique asymmetrical division mechanisms that give rise to two unequal daughters, one of which retains stem-cell identity and is able to repeat this process over and over again, whereas the other initiates differentiation.

The *Drosophila* testis has proven to be a useful tool to investigate stem cell biogenesis. It consists of an elongated coiled tube full of germ cells at different developmental stages, which include gonioblasts, mitotically dividing spermatogonia, primary and secondary spermatocytes and elongating spermatids. Two stem cell lines, germline stem cells (GSCs) and cyst progenitor cells (CPCs), are found at the apical tip of the testis, anchored by thin cytoplasmic extensions to a cluster of nondividing cells, the hub cells (Hardy et al., 1979; Fuller and Spradling, 2007). It has been proposed that each GSC is flanked in the niche by a pair of CPCs, forming a complex of three cells that divide in synchrony, perhaps following specific signals (Decotto and Spradling, 2005). The three daughter cells that remain in contact with the hub cells retain the stem cell fate, whereas the other three cells associate to form a cyst. Within these cysts, the cytoplasmic extensions of two squamous cyst cells form a thin, microtubule-rich shell that surrounds the gonioblast, which undergoes four rounds of mitotic divisions with incomplete cytokinesis. The spermatogonia differentiate in primary spermatocytes that enter meiosis, producing cysts of 64 haploid spermatids (Fuller, 1993). As the spermatids begin to elongate, the

cyst cells change their behaviour. One of these, the 'head cyst cell', remains associated with the apex of the spermatogenetic cyst, whereas the other, the 'tail cyst cell', elongates dramatically to envelope the sperm tails that reached a length of about 2 mm (Fabrizio et al., 2008). Much less is known about the fate of cyst cells after the process of sperm individualization.

How do stem cells accomplish asymmetrical divisions to self-renew and to generate appropriate numbers of differentiated progeny? Recent advances in *Drosophila* stem cell research have provided evidence that, in addition to signals from the niche (Tran et al., 2000; Kiger et al., 2001; Tulina and Matunis, 2001; Leatherman and DiNardo, 2008; Wang et al., 2008), the centrosomes have a precise role in the mechanism of spindle orientation (Lin, 2008; Yamashita and Fuller, 2008) that is crucial to establish the polarity of the stem cells within the niche. A striking observation is the preferential inheritance of centrioles within daughter cells: the mother always remains in the germline stem cell and daughters move into the forming gonioblasts (Yamashita et al., 2007). Misoriented centrosomes have also been involved in the cell-cycle arrest of GSCs, which leads to reduction of spermatogenesis during ageing in the *Drosophila* testis (Cheng et al., 2008). Proper spindle orientation might depend on the different microtubule-nucleating activity of the centrosomes. Accordingly, defects in the adenomatous polyposis coli (APC) tumor-suppressor protein, localized at the hub stem cell zonula adherens, and mutations in *centrosomin* (*cnn*), which affect an integral centrosomal protein and give rise to anastral poles, result in spindle misorientation and excess GSCs (Yamashita et al., 2003). Similar observations of centrosome behaviour in *Drosophila* neuroblasts (Rusan and Peifer, 2007; Rebollo et al.,

2007) suggest general mechanisms of spindle positioning and orientation during the asymmetrical divisions.

However, although a growing body of data is available on GSCs, the mechanisms underlying CPC division and cyst cell behaviour have received little attention and must still be unravelled. In particular, it is generally accepted that cyst cell daughters withdraw from the cell cycle, whereas the spermatogonia they surrounded divide again, but how this process occurs has not been yet clarified.

Results and Discussion

The cytoplasm of cyst cells is filled with a loose microtubule network, but contains no detectable astral arrays of microtubules. However, microtubule asters might escape detection because of the small size and the laminar shape of the cyst cells. We examined testes from the *Drosophila* mutant *nup154* in which the cyst cell pairs surrounded incomplete sets of germ cells and also formed when germ cells were lacking (Gigliotti et al., 1998). Under these conditions, the cyst cell cytoplasm was expanded, allowing us to examine microtubule distribution in more detail (Fig. 1A). We

Fig. 1. Centrosomes are gradually downregulated in cyst cells. (A) Detail of a *nup 154* mutant testis showing cyst cell pairs either associated with a few germ cells (arrow; Vasa, red) or alone; microtubules (green) form a thin network, but cytoplasmic asters are not visible. Nuclei are stained blue with Hoechst 33258. (B-D) Details of the testis tip of newly eclosed wild-type males labelled with antibodies against Vasa (red, germ cells), γ-tubulin (green in B), SPD2 (red in C), PLP (red in D), β-tubulin (green in C,D); nuclei are stained blue with Hoechst 33258. GSCs (gsc) and CPCs (cpc) undergoing mitosis are visible. Arrows indicate centrosomes of GSCs, arrowheads indicate centrosomes in cyst cells. (E-J) Details of wild-type spermatogonial cysts during second (E,H), third (F,I) and fourth (G,J) mitotic divisions labelled with antibodies against γ-tubulin (green, E-G) and Vasa (red) and with antibodies against PLP (red, H-J) and tubulin (green); nuclei are blue. Arrowheads indicate centrosomes in cyst cells (cc). Scale bars: 10 μm.

confirmed that cyst cells were devoid of microtubule asters, suggesting that centrosomes are missing or improperly working. To address this point, we followed centrosomal dynamics in both GSCs and CPCs and their immediate daughters, primary spermatogonia or nondividing cyst cells, by labelling larval, pupal and adult testes with antibodies against tubulin and several centrosomal markers. We also counterstained testes with the specific germ cell marker Vasa (Lasko and Ashburner, 1988), to unambiguously recognize cyst and germ cells in immunofluorescence preparations.

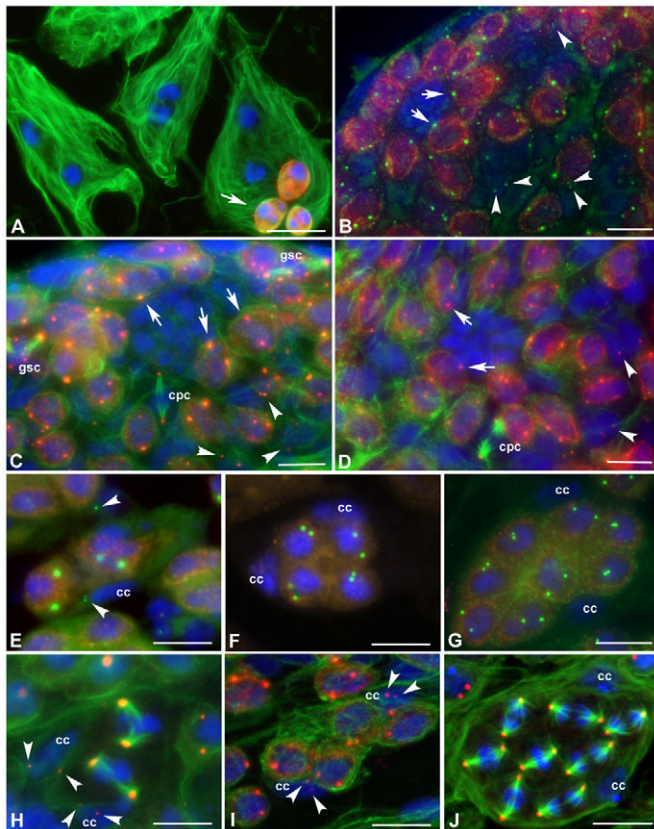### Centrosome function is downregulated in cyst cells

Functional centrosomes able to organize dynamic microtubule arrays were detected in GSCs, spermatogonia and spermatocytes (Fig. 1B-D). Centrosomes were also found in CPCs and their immediate daughters (Fig. 1B-D). Antibodies against γ-tubulin, CNN and CP190 (only γ-tubulin staining is reported here) recognized discrete centrosomes in the cyst cells that surround gonioblasts or spermatogonia during the second mitosis (Fig. 1E), but failed to stain distinct foci in older cyst cells (Fig. 1F,G). Staining for the centriolar markers PLP and SPD-2 (only the PLP labelling is reported here) was found in cyst cells associated with spermatogonia during the second (Fig. 1H) and third mitosis (Fig. 1I), but disappeared soon after (Fig. 1J).

This lack of centrosomes in the cyst cells that envelope spermatogonial cells during their last mitoses might be due to a loss of centrioles that have a key role in the organization and recruitment of the centrosomal material. Alternatively, centrioles might still be present, but unable to recruit pericentriolar material and to act as functional microtubule-organizing centers (MTOCs), as reported in some interphase *Drosophila* cells (Rogers et al., 2008). To test these hypotheses, we looked at the centriole cycle in CPCs by labelling the apical tip of the testis with an antibody against the centriolar marker *Drosophila* SAS-6 (Peel et al., 2007; Rodrigues-Martins et al., 2007). This antibody recognized distinct spots in GSCs (Fig. 2A) and revealed spot pairs at each pole of the mitotic spindle in dividing spermatogonia (Fig. 2B). These findings suggest a canonical centriole biogenesis in the germ cell lineage and indicate that these organelles undergo a cell-cycle-related replication process. Pairs of small SAS-6-positive dots were also observed at the spindle poles of dividing CPCs (Fig. 2C,D). The SAS-6 antibody indentified small spots in the cytoplasm of the cyst cells, whether they surrounded dividing spermatogonia (Fig. 2E) or primary spermatocytes (Fig. 2F). This suggests that centrioles are present in these cells despite the lack of pericentriolar material.
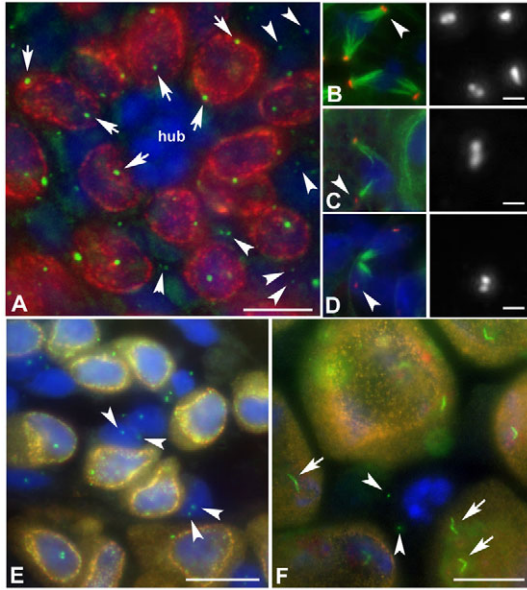
The centriole cycle is arrested in cyst cells

The finding of only two spots within the cyst cell cytoplasm suggests that either inherited centrioles are unable to duplicate, or that procentrioles are so small that they were difficult to identify by immunofluorescence.

To distinguish between these possibilities and to verify if cyst cells contained *bona fide* centrioles, we examined stem cell niche and germ cell cysts by EM (Fig. 3A). Germ cells and cyst cells can be distinguished at the EM level by their shape and electron density. Germ cells are rounded and linked together by large cytoplasmic bridges; cyst cells are laminar, with a wavy plasma membrane and a faintly opaque cytoplasm full of cisternae and vesicles. Centrioles were found in both stem (Fig. 3B,C) and hub (Fig. 3D) cells. Cells were considered to be CPCs on the basis of their cytoplasm density, nuclear shape and proximity to the hub. We identified only seven

Fig. 2. SAS-6 is maintained in cyst cells. (A) Detail of the testis tip of a newly eclosed male in which centrosomes (arrows) of the GSCs surrounding the hub, and smaller centrosomes of CPCs and cyst cells (arrowheads) are visible. Green, SAS-6; red, Vasa; blue, DNA. (B) Detail of dividing spermatogonia (B) and dividing CPCs during anaphase (C) and telophase (D); arrowheads indicate centrosomes at the spindle poles. Magnified panels on the right show separated centrioles at the spindle poles. Green, tubulin; red, SAS-6; blue, DNA. Centrosome labelling in cyst cells (arrowheads) associated with spermatogonia during the first mitosis (E) and with primary spermatocytes (F); note the large rod-like centriole pairs of the spermatocytes (arrows). Green, SAS-6 (centrioles); red, Vasa (germ cells); blue, Hoechst 33258 (DNA). Scale bars: 10 μm (A,E,F); 4 μm (B-D).

cells that met the requirements to be identified as CPCs, and only three of them contained cross-sections suitable for understanding centriole architecture. By contrast, GSCs were frequently encountered, and we scored 19 suitable images of cross-sectioned

centrioles. Both stem cell types had centrioles formed by nine peripheral triplets and a central tubule in a cartwheel arrangement (Fig. 3E,F). Hub cells had unusual centrioles with nine doublets and a large central tubule (Fig. 3G). Procentrioles were apparently lacking in serial sections of hub cells (n=7), suggesting that centriole duplication might be hampered. This is consistent with previous results indicating that hub cells are post-mitotic (Hardy et al., 1979; Gonczy and DiNardo, 1996).

The size of the procentrioles in the centrosomes of spermatogonia ranged from thin accumulations of electron-dense material at the base of the mother centriole (Fig. 3B) (n=11) to well developed centrioles that were similar to the mother (Fig. 3H) (n=9). The centrioles therefore undergo canonical maturation during spermatogonial mitoses. The whole centriole was crossed with a central tubule in spermatogonia (Fig. 3H) (n=8), whereas the tubule was restricted to the basal half of the more elongated centrioles in early spermatocytes (Fig. 3I) (n=13). Cross-sections confirmed the central tubule along the whole extent of the spermatogonial centriole (Fig. 3J) (n=15), whereas the tubule was restricted to the basal half of the centrioles scored in primary spermatocytes (Fig. 3K,L) (n=19).

Centrioles were associated with centrosomes that were able to organize functional asters during mitosis of CPCs (Fig. 2D), but we did not find astral microtubules in cyst cells associated with gonioblasts. This indicates that CPCs have functional centrosomes able to duplicate and organize a mitotic spindle, whereas cyst cell centrosomes do not. EM analysis failed to reveal procentriole maturation in cyst cells. Indeed, we observed that mother centrioles had started to replicate, but emerging procentrioles were always thin, suggesting that they did not elongate further. This scenario was present in cyst cells that surrounded both spermatogonia (Fig. 4A-E) (n=12) and primary spermatocytes (Fig. 4F) (n=7). Microtubules did not interact with the centrosomes in the cyst cell cytoplasm (Fig. 4B), suggesting that the pericentriolar material, if there was any, was not functional. Since canonical procentriole formation occurs in association with pre-existing centrioles at the G1-S transition in coordination with nuclear events (Hinchcliffe and Sluder, 2001), the cyst cells were expected to enter the DNA
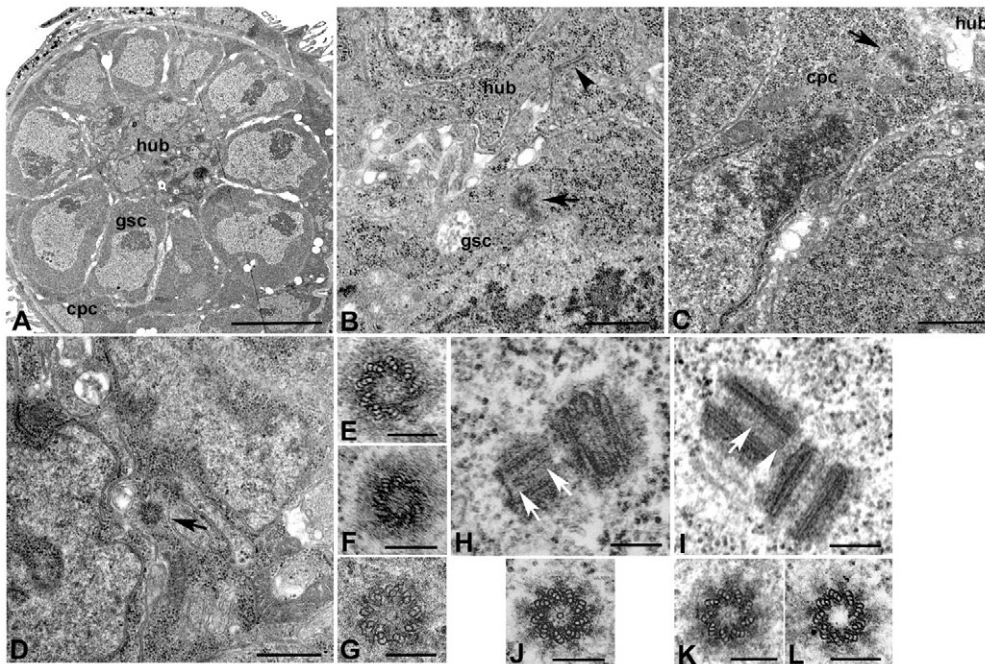
Fig. 3. Transmission electron microscopy images of centrioles in stem cells. (A) Cross-section through the apical tip of a newly eclosed male showing the hub region (hub), GSCs (gsc) and CPCs (cpc). Details of the apical region: a GSC (B, gsc), and a CPC (C, cpc) in contact with the hub cells (hub); arrows indicate centrioles; arrowhead indicates the *zonula adherens* between hub and GSC. (D) Cross-section of the hub with a centriole (arrow) visible. (E-G) Details of centrioles in GSCs, CPCs and hub cells, respectively: stem cell centrioles have nine triplets and a central tubule, whereas hub cell centrioles are formed by nine doublets and a larger central tubule. Longitudinal sections throughout centriole pairs in late-dividing spermatogonia (H) and early primary spermatocytes (I): white arrows indicate the extension of the central tubule within the centrioles. Cross-section of a spermatogonial centriole (J) and through the basal (K) and the distal (L) half of a spermatocyte I centriole. Scale bars: 10 μm (A); 1 μm (B-D); 0.1 μm (E-L).
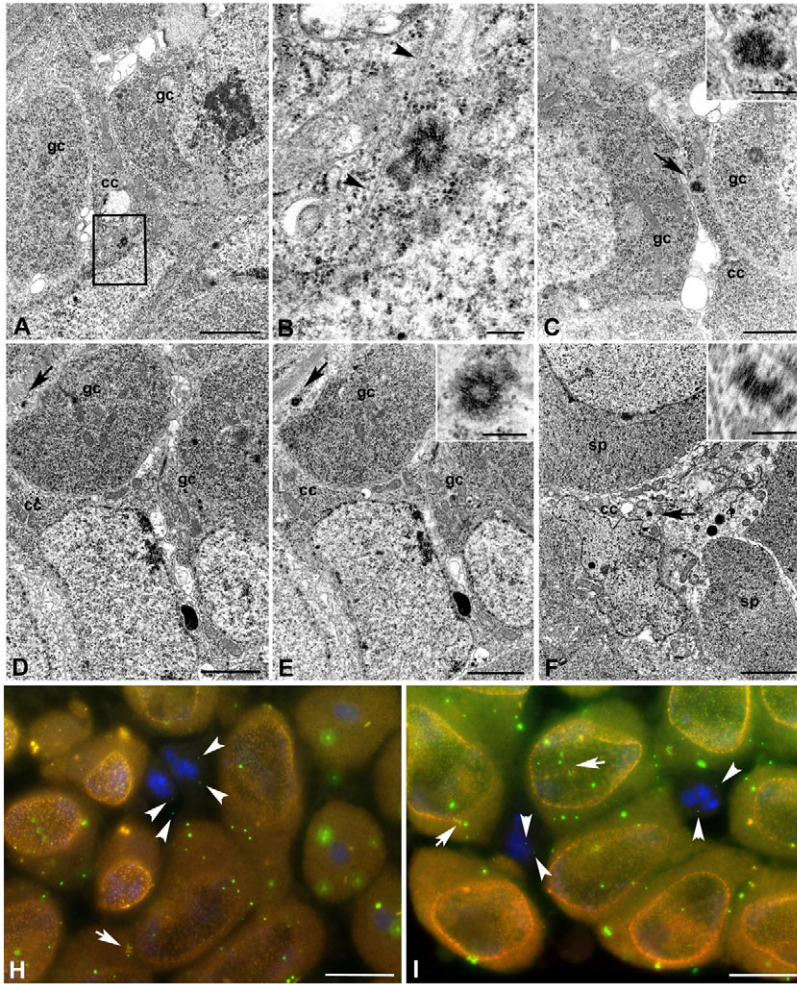
Fig. 4. Procentrioles in cyst cells do not elongate properly. Electron micrographs of cyst cells (cc) associated with spermatogonia (gc) during the first mitosis (A,B), third mitosis (C-E) and with primary spermatocytes (F,sp); arrows indicate centrioles in cyst cells. Note that microtubules (arrowheads in B) do not interact with centrosomes in cyst cells. Insets are magnifications showing thin procentrioles associated with the mothers. (D,E) are consecutive sections. (G,H) Details of spermatocytes in GFP-SAS-6-overexpressing testes; only two small spots are visible in cyst cells (arrowheads), whereas larger SAS-6 aggregates are found within the spermatocyte cytoplasm in addition to the centriole pairs (arrows). Scale bars: 3 μm (A,C-F); 0.1 μm (B, and insets in C,E,F); 10 μm (H,I).

duplication cycle. However, there are conflicting reports about whether centrosome duplication is uncoupled or not from DNA synthesis (Durcan et al., 2008). The failure of the somatic cyst cell lineage to incorporate BrdU (Voog et al., 2008), suggests an earlier developmental block, which thus represents a useful in vivo indication that centriole duplication in these cells is uncoupled from G1-S transition. This might start in G1, as reported in vertebrate cultured cells during mimosine-induced G1 arrest (Durcan et al., 2008), or even before in mitosis. Therefore, cell-cycle arrest might result in the improper accumulation of centriole components at the mother centriole, thus hampering the full elongation of its daughter. It has indeed been demonstrated that daughter centriole assembly in *C. elegans* embryos is promoted by the pericentriolar material (Dammermann et al., 2004). Accordingly, the overexpression of pericentrin in S-phase-arrested CHO cells induces the formation of several daughter centrioles (Lonkarek et al., 2008). It is also tempting to speculate that procentriole elongation defects result in defective recruitment of the centrosomal material or could lead to the inability to maintain centrosomal components during development. This possibility appears plausible, because human cells and *C. elegans* embryos with compromised centrioles are defective in the recruitment of pericentriolar material (Bobinnec et al., 1998; Kirkham et al., 2003). Loss of centrosome integrity in cyst cells could then lead to cell-cycle progression defects and failure to enter S-phase.

Centrosome-dependent cell-cycle arrest in G1, by activation of a checkpoint that prevents the entry into S phase, has been reported in human cultured cells following removal of pericentriolar proteins (Mikule et al., 2007). The finding that most aspects of the *Drosophila* development can take place without centrioles (Basto et al., 2006) does not support the hypothesis that defects of procentriole maturation are involved in the withdrawal of the cyst cells from the cell cycle. However, the centrioles appear to be necessary to maintain stem cell lineage in the *Drosophila* testis (Yamashita and Fuller, 2008).

SAS-6 is downregulated in cyst cells
The above findings led us to ask whether the block of the centriole cycle in cyst cells could be due to inappropriate levels of the proteins involved in the assembly of the centrioles. We next examined testes from a *Drosophila* strain overexpressing a SAS-6–GFP fusion protein (Rodrigues-Martins et al., 2007), one of the main regulators of centriole architecture. Stable expression of this protein does not increase centriole number, but leads to the formation of many aggregates of variable size in the cytoplasm of the germ cells that do not colocalize with centrosomal markers. These aggregates might be nonfunctional but might reflect the transcriptional activity of the cells. Their amount, indeed, noticeably grew in primary spermatocytes as protein synthesis increased (Fig. 4H,I). However, in cyst cells, only two spots of

GFP–SAS-6, that correspond with centrioles from EM analysis, were found (Fig. 4H,I). These observations indicate that SAS-6 might be downregulated after the asymmetrical division of the CPCs. Therefore, the cyst cell daughters might receive a small amount of SAS-6 protein from CPC mothers, which is enough to support the early process of centriole replication, but inadequate to promote the full elongation of the procentriole.

Materials and Methods

Drosophila strains
OreR stock was used as the wild type. *pUbq-GFP-SAS-6* and *pUASp-GFP-SAS-6* transgenic flies were kindly provided by Monica Bettencourt-Dias (Insitut Gulbenchian de Ciencias, Oeiras, Portugal). The gfp-nup154 transgenic fly strain was obtained from Silvia Gigliotti (Institute of Genetics and Biophysics, Naples, Italy). Flies were raised on standard *Drosophila* medium in 200 ml plastic containers at 24°C. Testes were dissected from older pupae, pharate adults and just emerged males.

Antibodies
We used the following antibodies: chicken anti-*Drosophila* SAS-6 [SAS-6, 1:1000, (Rodriguez-Martins et al., 2007)]; rat anti-α-tubulin-YL1/2 (1:50, Oxford Biosciences); mouse anti-β-tubulin (1:200, Boehringer); mouse anti-γ-tubulin-GTU88 (1:100, Sigma); rabbit anti-centrosomin (1:400) (Vaizel-Ohayon and Schejter, 1999); rabbit anti-Vasa (1:400) (Lasko and Aschburner, 1988); rabbit anti-SDP-2 (1:500) (Rodriguez-Martins et al., 2007); rabbit anti-CP190 (1:400) (Whitfield et al., 1988) and rabbit anti-*Drosophila* PLP (1:1500) (Martines-Campos et al., 2004). Anti-mouse, anti-rabbit, anti-rat and anti-chicken secondary antibodies linked to Alexa Fluor 488 or Alexa Fluor 555 (1:800), were obtained from Invitrogen.

Immunofluorescence preparations
To immunostain testes, we used the methanol-acetone fixation method (Glover and Gonzalez, 1993; Cenci et al., 1994). Briefly, testes were dissected in phosphate-buffered saline (PBS) and placed in a small drop of 5% glycerol in PBS on a glass slide. Testes were squashed under a small coverslip and frozen on a copper bar pre-cooled in liquid nitrogen. After removal of the coverslip, the squashed samples adhered to the slides were immersed in methanol for 10 minutes at –20°C and in acetone for 5 minutes at –20°C.

For localization of microtubules and centriole or centrosomal components, the frozen samples were fixed in methanol at –20°C. Testes were washed for 15 minutes in PBS and incubated for 1 hour in PBS containing 0.1% bovine serum albumin (PBS-BSA) to block nonspecific staining. The samples were incubated overnight at 4°C with the specific antisera against CP190, CNN, SPD-2, PLP, *Drosophila* SAS-6 and then with anti-β-tubulin antibody for 4-5 hours at room temperature. After washing in PBS-BSA the samples were incubated for 1 hour at room temperature with the appropriate secondary antibodies. In addition, to recognize germ cells from CPCs and their daughter cyst cells, testes were often incubated with the anti-Vasa antibody. Since antibodies against SPD-2, PLP, CNN and CP190 were also raised in rabbit, we saw centrosomes in the same channel as the Vasa antigen. However, the centrosomes were easily recognizable in cyst cells that lack Vasa labelling. Samples were then washed in PBS-BSA and incubated for 1 hour in the appropriate secondary antibodies. For simultaneous localization of γ-tubulin and α-tubulin, the frozen testes were incubated in antibodies against either GTU88 or YL1/2 followed by anti-mouse and anti-rat secondary antibodies. In all cases, DNA was visualized by a 3-4 minute incubation in Hoechst 33258. Testes were mounted in small drops of 90% glycerol in PBS.

Image acquisition and presentation
The fluorescent images were taken using an Axio Imager Z1 (Carl Zeiss) microscope equipped with an HBO 50-W mercury lamp for epifluorescence and with an AxioCam HR cooled charge-coupled camera (Carl Zeiss). Final projection of fixed testes images was done with Z-stack and extended focus modules (Carl Zeiss) after collection of ten optical sections at 0.2 μm intervals. Gray-scale digital images were collected separately and then pseudocolored and merged using Adobe Photoshop 7.0 software (Adobe Systems).

Transmission electron microscopy
Testes from old pupae and young (1- to 2-day-old) adult males were dissected in PBS and fixed for 2 hours at 4°C in 2.5% glutaraldehyde in PBS. After rinsing for 30 minutes in PBS, the material was post-fixed in 1% osmium tetroxide in PBS for 2 hours. After extensive washing in distilled water, the samples were stained en bloc for 1 hour in 1% uranyl acetate, dehydrated in a graded series of alcohols, and then embedded in an Epon-Araldite mixture and polymerized at 60°C for 48 hours. Ultrathin sections obtained with a Reichert Ultracut II E ultramicrotome were stained with uranyl acetate and lead citrate and observed with a TEM Philips CM 10 operating at 80 kV. Only centrioles in suitable cross-sections or longitudinal sections were considered for this study, and their number is reported in parentheses for each cell type examined.

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