Supplementary Texts I and II.

I. Centrin-GFP fusion protein is a reliable marker for early stages of centriole duplication.

Centrin is a small calcium-binding protein that localizes in the distal part of centriolar lumen from the earliest stages of centriole formation[1]. This property makes a centrin-GFP fusion[2, 3] an ideal marker for visualizing of centriole duplication in live cells. However, one difficulty in using fluorescence Light Microscopy (LM) to visualize the exact moment of daughter centriole formation is that the centriole is only 200 x 500 nm in size. This means that the distance between the distal ends of two fully grown (400-500 nm) orthogonally arranged centrioles can be reproducibly resolved with high numerical aperture (NA) objectives. However, the distance between the distal part of the mother centriole and a newly-formed daughter is at or below the resolution limit of LM. Thus, visual doubling of centrin-GFP spots that occurs only after the daughter centriole reaches a certain length cannot be considered a reliable criterion for the initial stages of daughter centriole formation. In fact, in HeLa cells centrin-labelled centrioles become visually doubled ~5 hrs before the cell enters mitosis which corresponds to late S or G2 (Video S1). However, it is well established that centriole duplication occurs at G1/S transition[4].

While evaluating long-duration time-lapse movies of HeLa cells that express centrin-GFP, we noticed a subtle but reproducible difference in the appearance of individual centrin-GFP spots, a difference that occurs several hours before the spots become visually doubled. Shortly after mitosis (in G_1), each centrin spot appears as a typical point-spread function, which is consistent with the small size of the centriole (Fig.S1A). Then, approximately 8 hr after mitosis, centrin spots become asymmetric, developing a “shadow”

on one side. Same-cell correlative LM / serial-section electron microscopy (EM) analyses demonstrate that these shadows correspond to procentrioles situated next to the mother centriole (Fig. S1B). In fact, we find that procentrioles as short as ~150 nm can be reproducibly detected in live cells via such a distortion of the centrin-GFP spot (Fig.S1B). As the length of the daughter centriole increases, the distortion becomes more pronounced; finally, the mother and daughter centrioles can be seen as two adjacent centrin spots when the length of the daughter exceeds 250 nm (13 cells analyzed). Thus, centrin-GFP is a reliable marker for centriole duplication.

In the classical diplosome the daughter centriole is oriented orthogonal to the mother and is situated near the mother's proximal end[5]. However, in 3 of 12 centrosomes (in 6 cells) we found short procentrioles that were not oriented orthogonal to the mother (Fig. S2). Further, in one centrosome the procentriole was found near the end of the mother that bears distal appendages. Longer daughter centrioles (>200 nm) were at their expected positions and were properly oriented, in all examined cells (Fig. S1B). Although the low sample size of our EM reconstructions is a limitation, these findings are compatible with the idea that the rigid organization of the diplosome is established only after the procentriole is formed and reaches a certain length. It is noteworthy that the lower stringency of the orientation of procentrioles relative to the longer daughter centrioles has been described in the literature (see Fig.1 in ref.[6], and Fig. 3 in ref.[7]).

II. Dynamics of centriole duplication in HeLa and CHO cells treated with 2-mM hydroxyurea.

It has been demonstrated that HeLa cells posses a stringent mechanism that prevents centriole reduplication even when these cells are arrested during S phase[8]. We

evaluated the dynamics of centriole duplication in the centrin-GFP-expressing sub-clone of HeLa cells that was used in our study.

Mitotic HeLa cells were collected by shake-off from asynchronous untreated populations and were plated on coverslips in full growth medium supplemented with 2-mM hydroxyurea (HU). The first signs of centriole duplication were detected 7-8 hr after mitotic shake-off. At this time, most cells contained two individual centrioles, while in ~10% of cells centrioles began to develop centrin-GFP 'shadows'. By 12 hr, most cells contained two diplosomes. This configuration persisted until 72-73 hr (end of observations). Thus, our data confirm that mother and daughter centrioles remain engaged, and that centriole reduplication does not occur in HeLa S-arrested HeLa cells (Fig. S3A).

In marked contrast to HeLa cells, CHO-K1 cells have been shown to gradually accumulate supernumerary centrioles when they are arrested during S[9]. Mother and daughter centrioles disengage in these cells; however, it is not known whether all centrioles duplicate upon disengagement[9], or whether mothers reduplicate while the daughters remain immature[10]. We followed the process of centriole duplication in a sub-clone of CHO-K1 cells that expresses centrin1-GFP (Methods).

Mitotic cells were collected by shake-off and were plated in full growth medium supplemented with 2-mM HU. Time-lapse microscopy and fixed-cell analyses revealed that first round of centriole duplication occurred 6-7 hrs after mitosis (Fig. S3B). Approximately 24 hr after mitosis, mother and daughter centrioles began to disengage. Several hours after disengagement, both mother and daughter centrioles underwent another round of replication (Video S2), later followed by another disengagement, and yet another replication (not shown). In contrast to the first replication cycle, during which both mother centrioles in a cell had replicated at the same time, subsequent rounds of replication were progressively less synchronous. Replication of mother centrioles usually preceded that of the daughters

(Fig. 2). In some cases, daughter centrioles did not replicate until the second re-duplication of the mother within the same cell.

As evident from Videos S1 and S2, it was difficult to detect the exact moment of centriole duplication in the continuous long-term movies that had to be recorded at extremely low excitation light levels to avoid potential photo-damage to the cells. To circumvent this problem we developed a strategy where cells were followed at low temporal resolution and then shorter, high temporal-resolution time-lapse movies were recorded at times when centrioles were expected to duplicate (Video S3).

It has been suggested that the initial "seeds" of new centrioles can form in the cytoplasm and then become attached to the mother centriole[11, 12]. In fact, formation of numerous small aggregates of centrin-GFP can often be observed in cells during S phase. However, these aggregates usually form during late S period when centrioles are already duplicated (video S1). We also observed accumulation of small centrin aggregates in some CHO cells after prolonged exposure to hydroxyurea. These aggregates were not associated with other centrosomal components, such as γ-tubulin, however. EM microscopy revealed that the aggregates did not correspond to morphologically recognizable centrioles (not shown). We also noticed that extensive accumulation of centrin aggregates in HU-treated CHO cells occurred more often when cells were stressed, for example, in overly confluent cultures or when cells were kept in the same culture media for several days (not shown). Further, centriole reduplication occurred with similar dynamics in cells that contained numerous small centrin dots and in cells that completely lacked them. In every instance where we were able to record the exact moment of centriole duplication we did not detect incorporation of external centrin-GFP dots into the centrosome. Instead, the replicating centrioles developed a small centrin shadow that gradually transformed into a larger daughter centriole (Video S3). Thus, it is unlikely that accumulation of centrin-GFP

aggregates in the cytoplasm reflects activation of centriole de novo assembly pathway in S-phase arrested CHO cells[12].

Intriguingly, 5-10% of reduplicating centrioles simultaneously developed two daughters, thereby forming “triplosomes” (Figs. 2, S3B). This configuration was observed only for mother centrioles during their second and third rounds of duplication (28-60 hr after mitosis) and never in the first round. Triplosomes could be formed by both mothers (Video S4) or by just one of the two mothers present in the cell (Fig.2, Video S5). Occasionally, more than two daughter centrioles assembled in association with a single mother (Fig. S5). Multiple daughters were usually arranged in a ‘flower petal’ configuration forming centriolar ‘rosettes’. This configuration has been previously described in cells expressing Plk4[13] or Sas-6[14,15]. It is noteworthy, that the 3-D distribution of centrin-GFP within centriolar rosettes revealed that the daughters are positioned at different heights along the length of the mother centriole (Fig. S5).

Thus, centrioles in CHO cells arrested in S progress through repetitive replication and disengagement, although these cycles are often longer that the duration of a normal cell cycle in the untreated CHO cells. Furthermore, while the first centriole duplication, upon cell’s entry into S phase, is highly synchronous, consecutive rounds become progressively asynchronous indicating that duplication of individual centrioles is regulated by factors intrinsic to the individual centrosome rather than by global mechanisms acting on the whole cell.

References

1. Paoletti,A., Moudjou,M., Paintrand,M., Salisbury,J.L., & Bornens,M. Most of centrin in animal cells is not centrosome-associated and centrosomal centrin is confined to the distal lumen of centrioles. *J. Cell Sci.* **109**, 3089-3102 (1996).

2 Piel,M., Meyer,P., Khodjakov,A., Rieder,C.L., & Bornens,M. The respective contributions of the mother and daughter centrioles to centrosome activity and behavior in vertebrate cells. *J. Cell Biol*. **149**, 317-330 (2000).

3. White,R.A., Pan,Z., & Salisbury,J.L. GFP-centrin as a marker for centriole dynamics in living cells. *Microscopy Res. & Tech*. **49**, 451-457 (2000).

4. Hinchcliffe,E.H. & Sluder,G. "It takes two to tango": understanding how centrosome duplication is regulated throughout the cell cycle. *Genes & Development* **15**, 1167-1181 (2001).

5. Vorobjev,I.A. & Chentsov,Y.S. Centrioles in the cell cycle. I. Epithelial cells. *J. Cell Biol*. **93**, 938-949 (1982).

6. Kuriyama,R. & Borisy,G.G. Centriole cycle in Chinese hamster ovary cells as determined by whole-mount electron microscopy. *J. Cell Biol*. **91**, 814-821 (1981).

7. Pelletier,L., 'Toole,E., Schwager,A., Hyman,A.A., & Muller-Reichert,T. Centriole assembly in Caenorhabditis elegans. *Nature* **444**, 619-623 (2006).

8. Balczon,R.C. Overexpression of cyclin A in human HeLa cells induces detachment of kinetochores and spindle pole/centrosome overproduction. *Chromosoma* **110**, 381-392 (2001).

9. Balczon,R. et al. Dissociation of centrosome replication events from cycles of DNA synthesis and mitotic division in hydroxyurea-arrested Chinese hamster ovary cells. *J. Cell Biol*. **130**, 105-115 (1995).

10. Sluder,G. & Rieder,C.L. Controls for centrosome reproduction in animal cells: issues and recent observations. *Cell Motil. &Cytoskeleton* **33**, 1-5 (1996).
11. La Terra,S. et al. The de novo centriole assembly pathway in HeLa cells: cell cycle progression and centriole assembly/maturation. *J. Cell Biol*. **168**, 713-720 (2005).
12. Kuriyama,R., Terada,Y., Lee,K.S., & Wang,C.L. Centrosome replication in hydroxyurea-arrested CHO cells expressing GFP-tagged centrin2. *J. Cell Sci*. **120**, 2444-2453 (2007).
13. Kleylein-Sohn,J. et al. Plk4-induced centriole biogenesis in human cells. *Dev. Cell* **13**, 190-202 (2007).
14. Duensing,A. et al. Centriole overduplication through the concurrent formation of multiple daughter centrioles at single maternal templates. *Oncogene* **26**, 6280-6288 (2007).
15. Strnad,P. et al. Regulated HsSAS-6 levels ensure formation of a single procentriole per centriole during the centrosome duplication cycle. *Dev. Cell* **13**, 203-213 (2007).

Supplementary Figures

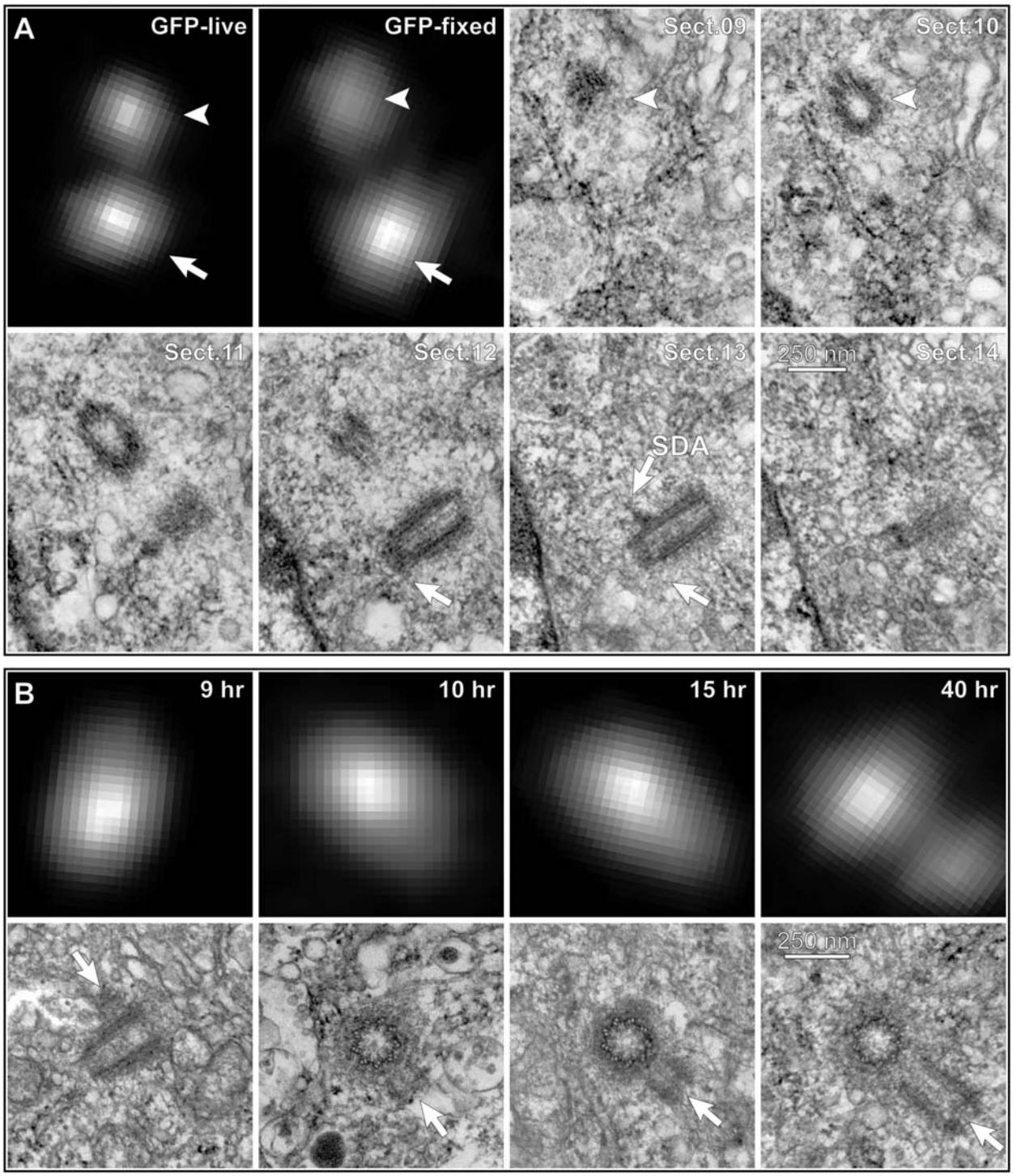

Figure S1. Centrin-GFP is a reliable live-cell marker of centriole replication. **(A)** Typical centrosome in a HeLa cell during G_1. In the live cell both centrioles appear as round spots

of centrin-GFP. One of the spots is consistently brighter than the other ("GFP-live" frame). Fixation with 2.5% glutaraldehyde results in only minor degradation of the image which occurs primarily due to the increase in non-specific green fluorescence in the cytoplasm (an effect which decreases the signal-to-noise ratio). However, it was not unusual for the centrioles to slightly change their orientation during the time necessary for fixation (cf. "GFP-live" and "GFP-fixed" frames). This change is likely due to the high mobility of centrioles in the cytoplasm. Both GFP images are maximal-intensity projections of a Z-series through the entire centrosome (200-nm steps). Serial-section EM analysis of the same cell revealed that the brighter centrin-GFP spot corresponded to the mother centriole (arrow in each frame) as evidenced by the fact that this centriole bears sub-distal appendages (labelled SDA in "sect.13" frame). The dimmer centrin-GFP spot corresponds to the daughter centriole (arrowhead in each frame). **(B)** Changes in the appearance of centrin-GFP spots during centriole replication. Maximal intensity projections of GFP-fluorescence series (200-nm steps) recorded immediately after fixation are shown in the top frame of each pair while the bottom frame presents a single EM section (80-nm) selected from the complete series through the same centrosome. Cells with the minimal distortion of the centrin-GFP spots contained 50-70-nm "procentrioles"; these appear as electron-dense material lacking developed microtubule triplets (arrow in lower "9 hr" frame). Daughter centrioles as small as ~150 nm create a prominent distortion of the centrin-GFP spot (arrow in lower "10 hr" frame). At later stages, centrin-GFP distortions become even more pronounced as daughter centrioles elongate (arrow in lower "15 hr" frame). Finally, the centrin-GFP spots become resolvable when daughter centrioles reach lengths of >250 nm (arrow in lower "40 hr" frame). The time shown on each frame reflects when each individual cell was fixed (after mitotic shake-off).

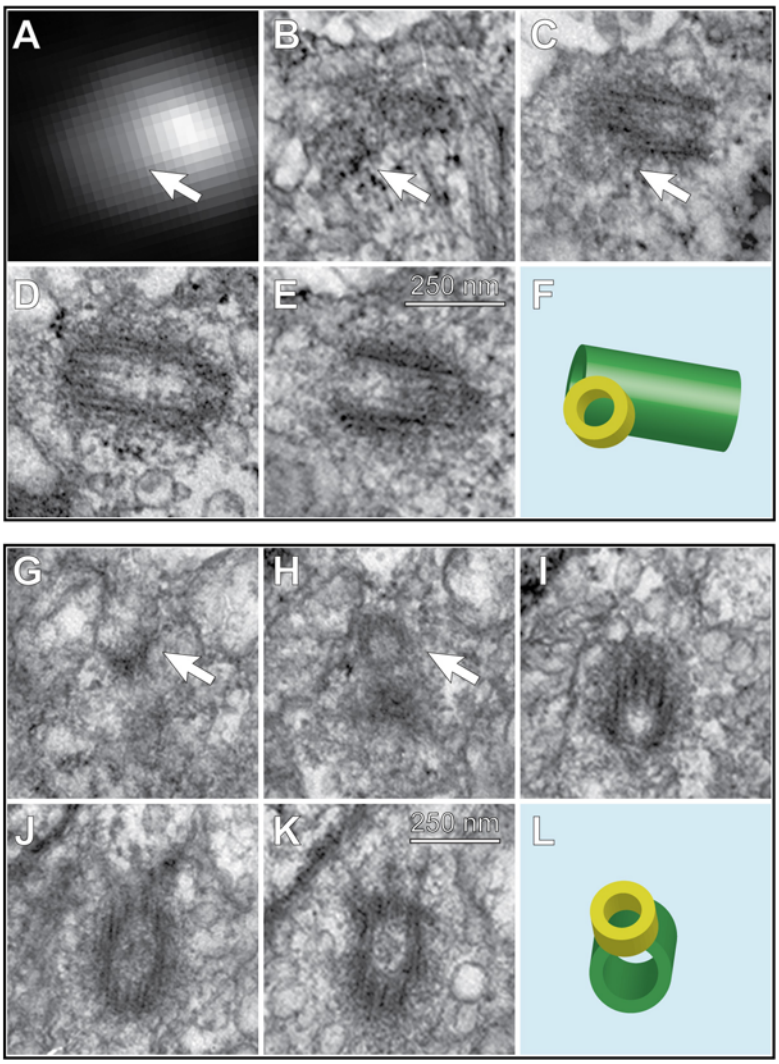

Figure S2. Procentrioles are not always oriented orthogonal to the mother centriole. Two examples (A-F and G-L) of early stages of centriole replication are shown. This stage of procentriole development is manifested by a slight distortion in the appearance of centrin-GFP spots (arrow in A). Such a distortion consistently corresponds to short (<100 nm) procentrioles (arrows in B-C and G-H). Surprisingly, young procentrioles are positioned at random angles with respect to mother centrioles (schematized in F and L).

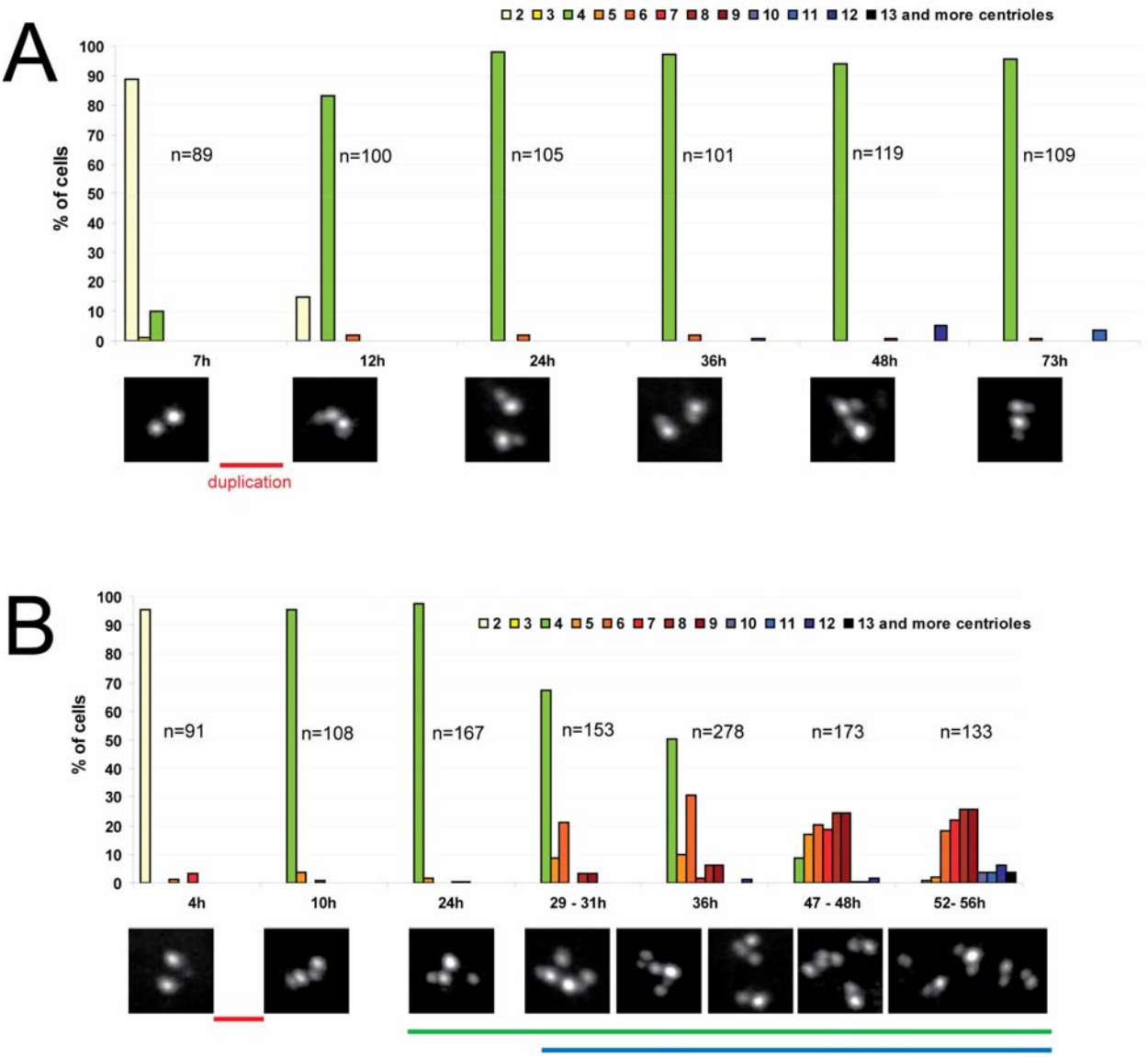

Figure S3. Dynamics of centriole duplication in S-arrested HeLa and CHO cells. Mitotic cells were collected by shake-off from untreated cultures and plated on coverslips in full growth medium supplemented with 2-mM hydroxyurea. Complete Z-series of centrin-GFP fluorescence were collected in live cells. Polyploid cells (recognized by exceedingly large nuclei and/or multiple nuclei) were excluded from analyses. Plots present percentages of cells with different numbers of centrioles in HeLa **(A)** and CHO **(B)** cells; typical centriolar configurations at the various time points are also shown (time after mitotic shake-off).

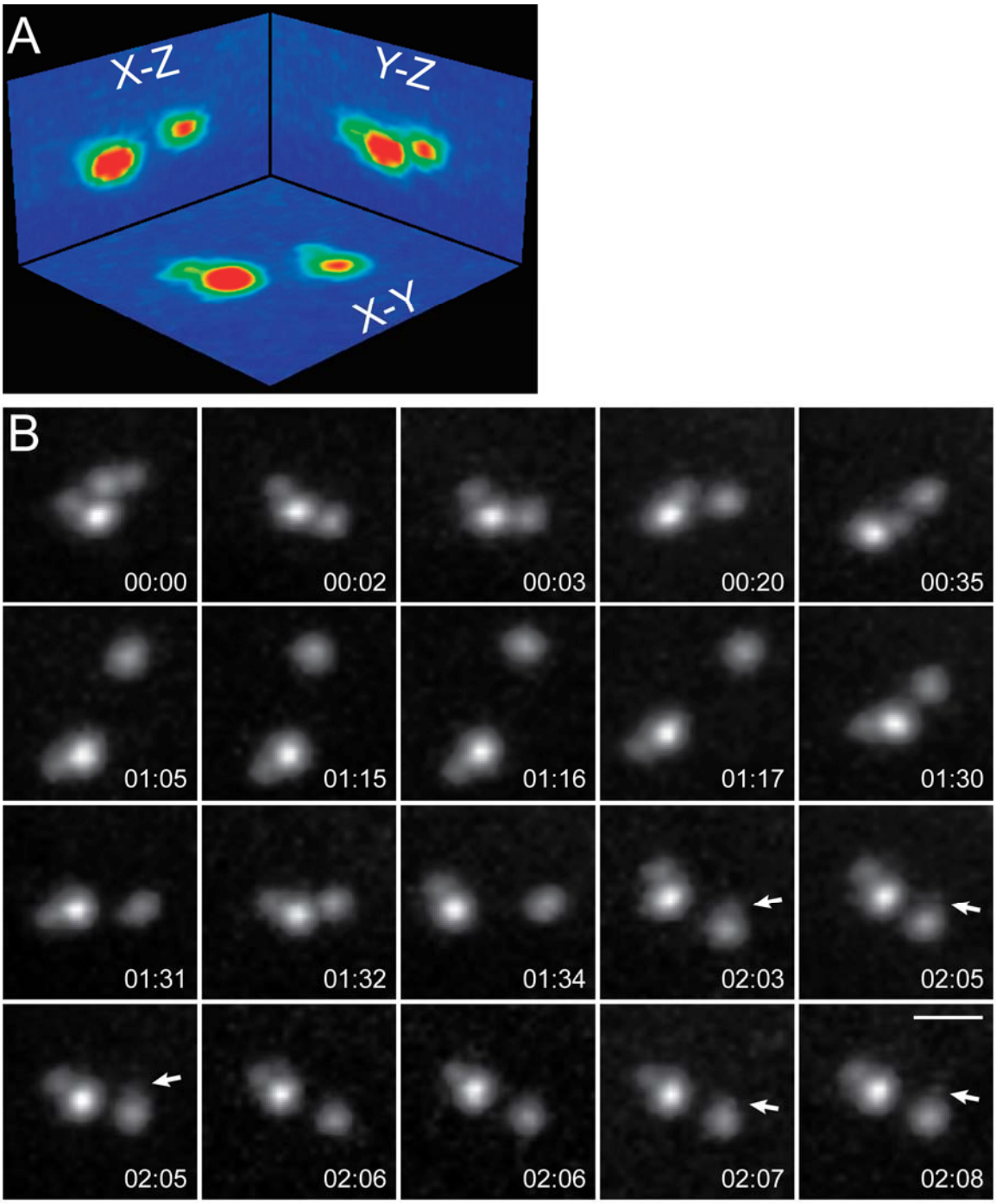

Figure S4. Complete set of fluorescence images illustrating the experiment presented in Fig. 1A. **(A)** Daughter centrioles can sometimes be masked by their mothers in planar projections. Notice that in this 3-D reconstruction (computed from the dataset presented in panel B "02:08") daughter centrioles in both diplosomes are clearly seen in the XY

projection. However, only one of the daughters is seen in the YZ, and none in the XZ projection. This implies that the ability to detect the initial stages of daughter centriole formation depend on the orientation of the diplosome. **(B)** To circumvent problems associated with unfavourable orientation of centrioles we collected several fluorescence datasets (30-60 seconds apart) at a single time point. Because centrioles continuously tumble in live cells, these multiple datasets allowed us to record the same diplosome in different orientations. Notice that the newly formed daughter centriole (arrows) is seen in five of seven datasets recorded at 2 hours after ablation of the original daughter. Time in hours : minutes. Scale bar represents 1 µm.

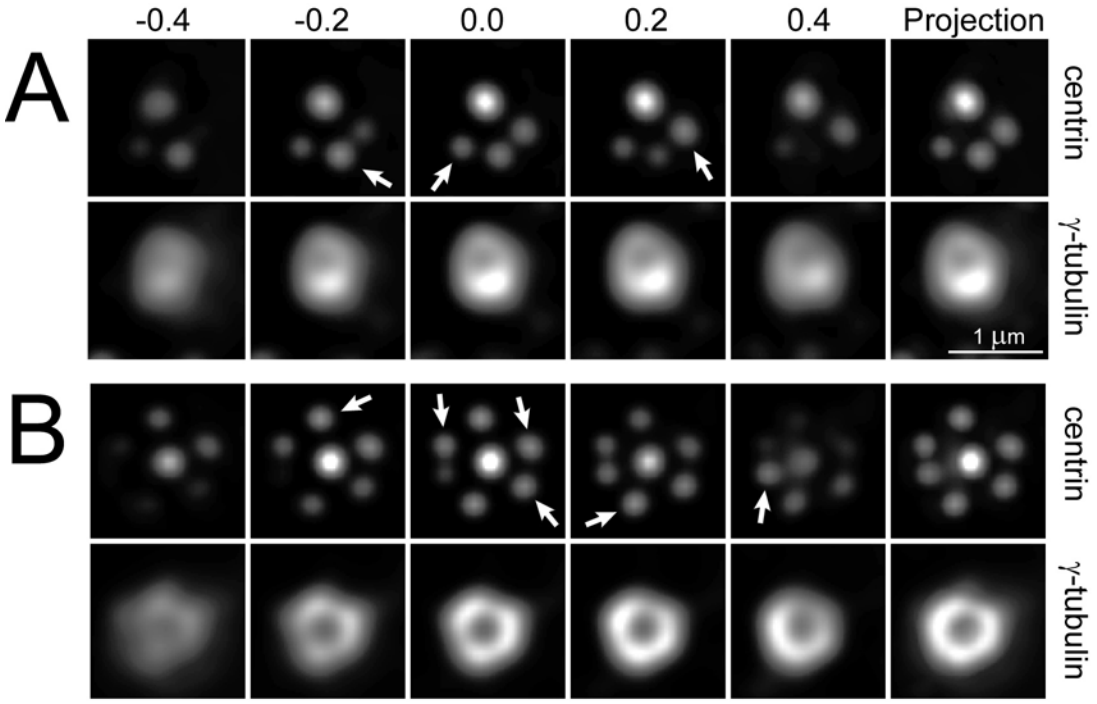

Figure S5. Organization of centriolar rosettes in CHO cells. **(A)** In this example four daughter centrioles form simultaneously in association with a single mother. Arrows indicate maximal intensities of centrin-GFP fluorescence of each daughter centriole. The middle Z-plane (0.0 µm) corresponds to the maximal intensity of centrin-GFP in the mother centriole.

(B) another example of multiple daughter centrioles associated with a single mother. In this case there are 6 daughter centrioles. Notice that the 3-D distribution of centrin GFP in the daughter centrioles indicates that different daughters reside at different heights along the mother centriole.

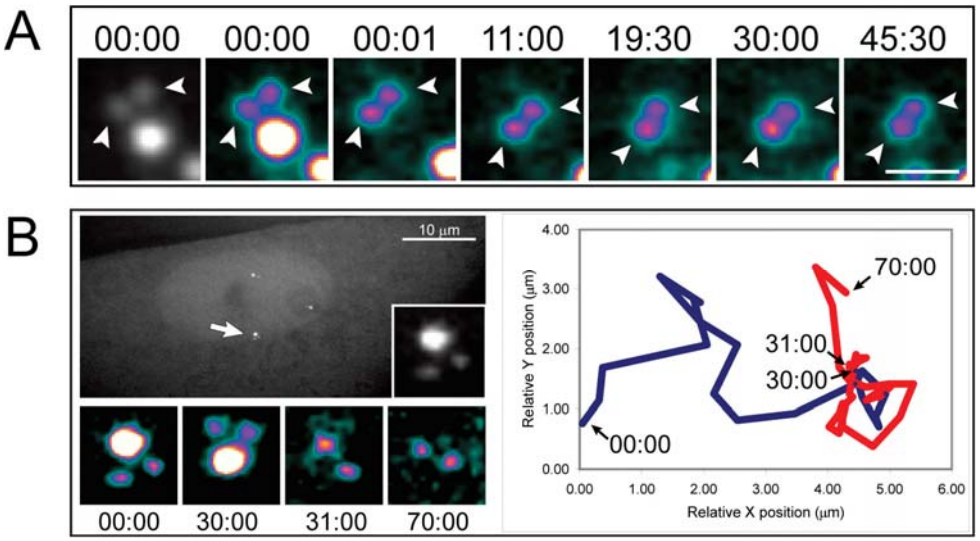

Figure S6. Daughter centrioles remain connected after ablation of the mother. **(A)** Example of mother-centriole ablation in a triplosome. The two daughters (arrowheads) remained in a common complex for ~45 min, at which point the cell was fixed. Serial-section EM analysis confirmed that the mother centriole was destroyed (not shown). **(B)** Another example of mother centriole ablation in a triplosome. In this cell, the centrioles did not form a common complex in the centre of the cell. Instead, individual diplosomes and a triplosome (arrow and inset in the first image) moved extensively through the cytoplasm. The trajectory of the triplosome is depicted by the blue line in the graph on the right. 30 min after the initiation of time-lapse recording, the mother centriole was ablated (cf. 30:00 and 31:00 frames). The remaining daughters continued to move in the cytoplasm (red line) as a single common complex for at least 40 min (cf. 31:00 and 70:00 frames). Scale bar in A = 1 µm. Same LUT as in Fig.1. Time stamps in minutes : seconds.

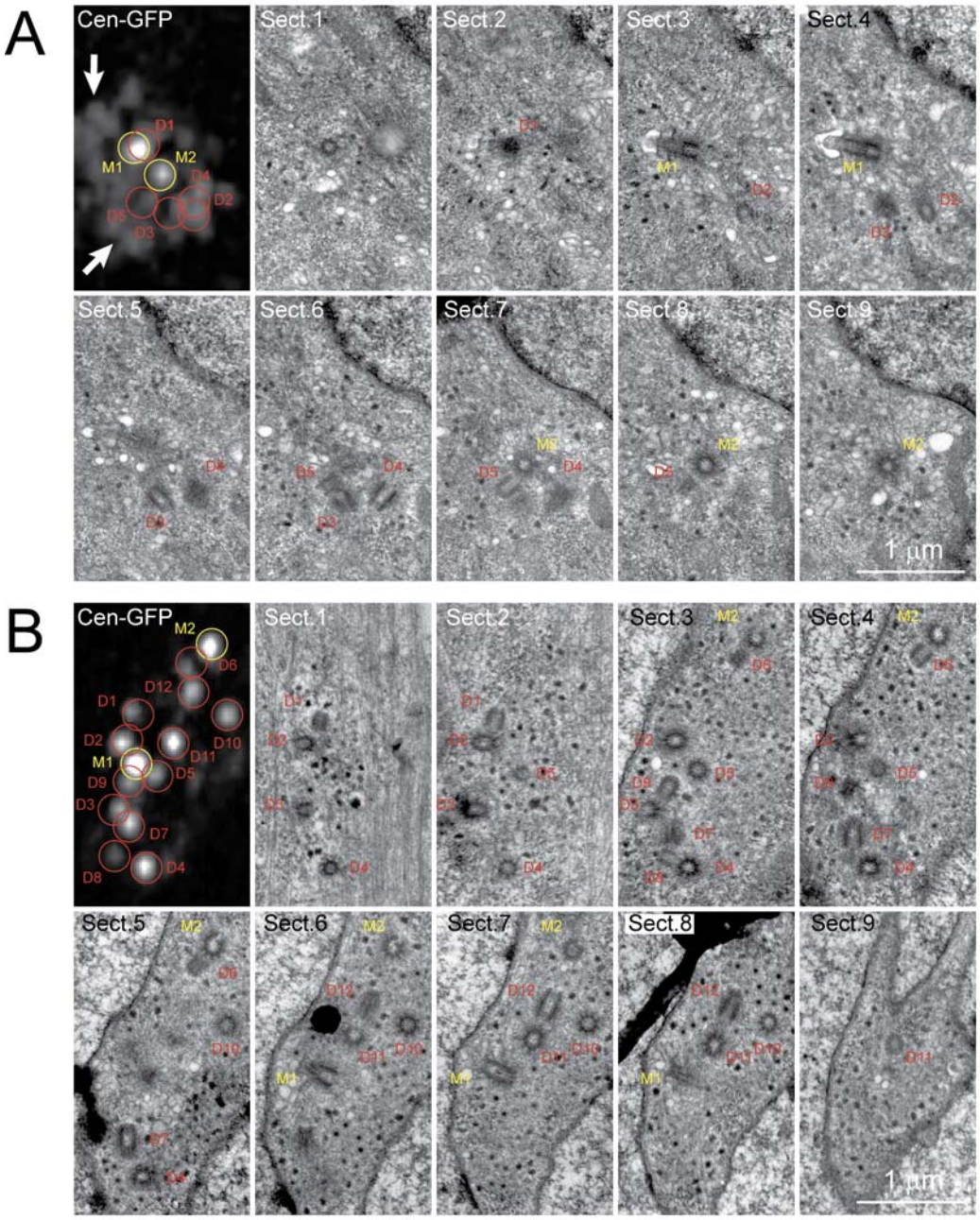

Figure S7. Many of centrin-GFP aggregates that form in S-arrested CHO cells upon overexpression of pericentrin are centrioles. Maximal-intensity projection of a 3-D fluorescence dataset (first frame) and serial 80-nm EM sections of two cells fixed 25 hr (A) and 45 hr (B) after transfection. See text for details.

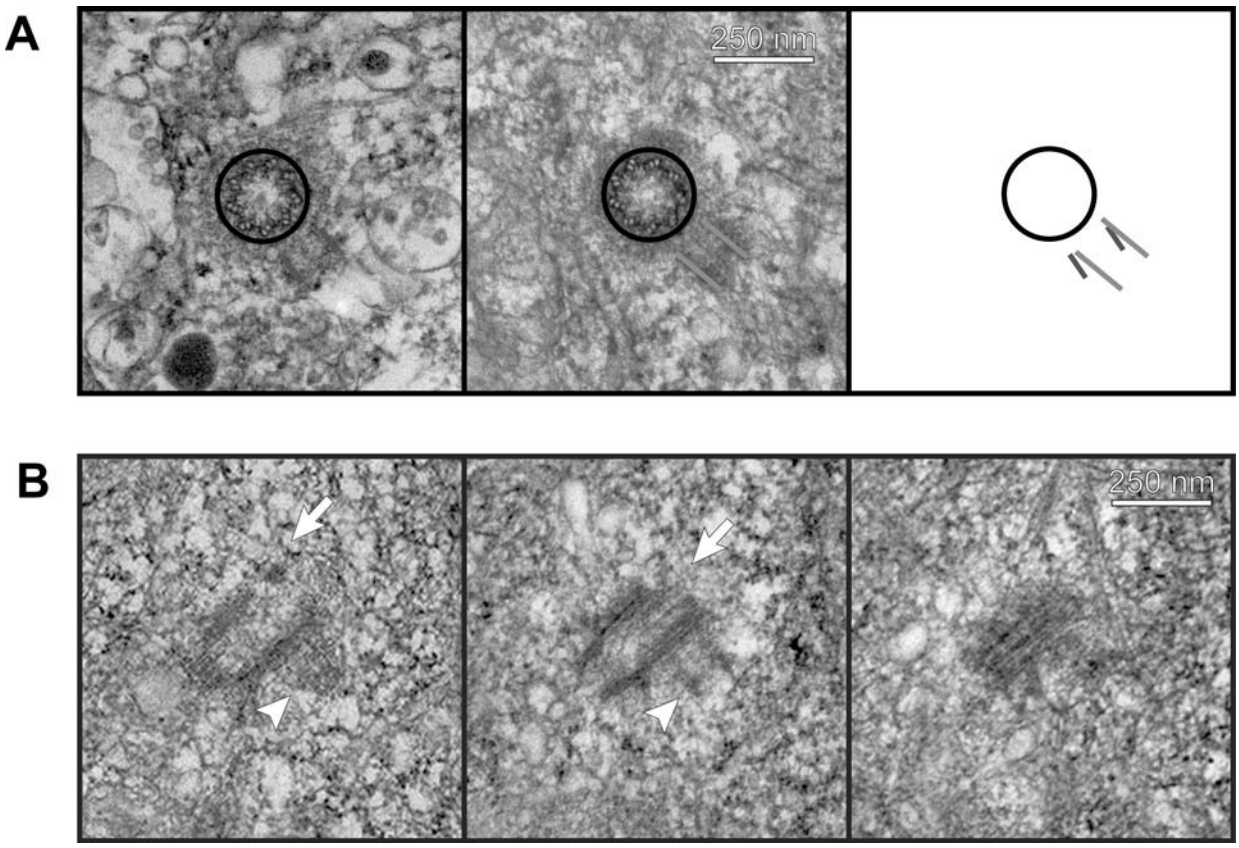

Figure S8. Additional data that suggest that daughter centrioles are not stringently attached to the wall of their mother. **(A)** The radial orientation of daughter centrioles, with respect to microtubule triplets in the mother, varies among individual diplosomes. Two diplosomes in different HeLa cells (also shown in Fig. S1B). Mother centrioles are rotationally aligned (notice position of the triplets). Positions of daughter centrioles are marked with coloured lines. Notice that because of the 9-fold radial symmetry of the centriole, the maximum variability in radial orientation of daughter centrioles cannot exceed $\pm 20^{o}$ because 40^{o} rotation will place the daughter on the next microtubule triplet in the mother. The angle between the radial positions in the two diplosomes shown is 17^{o}. **(B)** An example of a "nick" (arrow) on the mother centriole, inflicted by the laser beam during ablation of the daughter in a HeLa cell. Notice that the new daughter centriole (arrowhead) is attached to the mother on the side opposite from the nick.

Legends for Supplementary Videos

Video S1. Centriole behaviour during normal cell cycle in HeLa cells expressing centrin-GFP. The movie begins 100 min before mitosis and then follows one of the two daughter cells as it progresses through the cell cycle and into the next mitosis ~20 hrs later. Notice that the centrioles continuously move in the cytoplasm at all stages of the cell cycle. The mother centriole is consistently brighter than the daughter. Both centrioles become visually doubled ~5 hrs before the second mitosis (~900 min time point). Time in minutes; time 0 corresponds to the completion of the first mitosis. 5-min intervals, each frame is a maximal-intensity projection of the complete Z-series.

Video S2. Centriole reduplication in S-phase arrested CHO cells. The movie begins when both mother centrioles have undergone first round of duplication so that the cell contains two diplosomes. Centrioles in one diplosome disengage ~5 hr and the second diplosome breaks down ~8.5 hr after the beginning of the movie. All centrioles subsequently duplicate although it is not possible to point out the exact moment of duplication for each centriole in this movie which was recorded at a low intensity of the excitation light. Time in hours; time 0.0 corresponds to 20 hrs after mitotic shake off and addition of 2-mM hydroxyurea. 30-min intervals, each DIC frame is a single Z-plane, each fluorescence frame is a maximal-intensity projection of the complete Z-series.

Video S3. Reduplication of mother centrioles in S-phase arrested CHO cell. High temporal-resolution time-lapse recording that depicts the moment of centriole reduplication (~30 hr after mitotic shake off). Both mother centrioles (brighter spots) in this cell have duplicated once and disengaged from their daughters (dimmer spots). Notice that the mother and daughter centrioles in the centre of the frame 0.0 min reside very close to each other.

However these centrioles are not engaged as evident from their uncoordinated movements. The mother centriole in the centre of the frame develops a shadow that gradually transforms into a daughter centriole in ~30 min time period. The second mother centriole also reduplicates during this period and develops a clearly visible shadow by the “24.5 min” time point. Individual frames in this movie are aligned so that the mother centriole in the centre appears to be stationary. Each frame is a maximal-intensity projection. Fluorescence intensity is colour-coded using the lookup table presented in Fig.1.

Video S4. Movements of triplosomes and individual centrioles. Time-lapse recording of an HU-treated CHO cell (~ 47 hr after mitotic shake-off; 30-s interval; each frame is a maximal-intensity projection of the complete Z-series). During reduplication, both mother centrioles in this cell each developed two new daughters, forming ‘triplosomes’. Neither of the first-generation daughters has duplicated.

Video S5. Movements of diplosomes and triplosomes. Similar to Video S4, except that in this CHO cell, one mother centriole formed a single daughter, while the second mother centriole simultaneously developed two daughters. Both first-generation daughters also duplicated, forming diplosomes. Notice that movements of centrioles within the diplosomes and the triplosome are coordinated.

Video S6. The mother centriole and the two daughter centrioles within a triplosome are rigidly interconnected. Time-lapse recording of a triplosome in a CHO cell (60-s intervals; each frame is a maximal-intensity projection of the complete Z-series). Individual frames in this movie were aligned with respect to the mother centriole, which thus appears stationary in the movie. Daughter centrioles appear to move circumferentially around the mother centriole, an effect which is likely to be due to rotation of the entire centrosome. However,

the distances between the mother and each of the daughters, as well as between the daughter centrioles, remain constant (within the resolution of the recording). Pseudo-colour intensity lookup table, same as in Fig. 1.

Video S7. Uncoordinated movements of individual adjacent centrioles. Similar to Video S5, except that this movie presents a single centriole that is in close spatial proximity to a diplosome, in a CHO cell. Although in individual frames such a configuration may resemble a triplosome, the lack of coordination between the movements of the diplosome and the single centriole becomes apparent in the video. The sequence is aligned with respect to the mother centriole in the diplosome. Pseudo-colour intensity lookup table, same as in Fig. 1.

Video S8. Movements of daughter centrioles remain coordinated after ablation of the mother centriole in the triplosome. Individual frames are maximal intensity projections. The first frame presents a triplosome in a CHO cell immediately before laser irradiation. At this time, daughter centrioles were in the same focal plane which corresponds to the maximal apparent distance between two objects in a projection. Although the apparent distance between daughter centrioles appears to decrease in some frames, this effect is due to tumbling of the centrosome in 3D. The fact that separation between the centrioles never exceeds the original distance (indicated by yellow circle) reveals that they remain connected. The sequence is aligned with respect to the brighter daughter centriole. Pseudo-colour intensity lookup table, same as in Fig. 1.

Videos S9 – S11. Proximal ends of daughter centrioles in triplosomes (Videos S9 and S10) or in a large cloud of PCM induced _consistently reside within the cloud of γ-tubulin (PCM). Surface-rendered models presented in these movies represent centrosomes also shown in Fig.4D. Centrin-GFP which marks the distal end of centriole is shown in the green, SAS-6

which marks the proximal end of daughter centrioles is shown in the red, and γ-tubulin (PCM) is shown as isosurface corresponding to 25% of the maximal signal.