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De novo formation of centrosomes in vertebrate cells arrested during S phase

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The centrosome usually replicates in a semiconservative fashion, i.e., new centrioles form in association with preexisting "maternal" centrioles. De novo formation of centrioles has been reported for a few highly specialized cell types but it has not been seen in vertebrate somatic cells. We find that when centrosomes are completely destroyed by laser microsurgery in CHO cells arrested in S phase by hydroxyurea, new centrosomes form by de novo assembly. Formation of new centrosomes occurs in two steps: ~5–8 h after ablation, clouds of pericentriolar material (PCM) containing γ-tubulin and pericentrin appear in the cell. By 24 h, centrioles have formed inside of already well-developed PCM clouds. This de novo pathway leads to the formation of a random number of centrioles (2–14 per cell). Although clouds of PCM consistently form even when microtubules are completely disassembled by nocodazole, the centrioles are not assembled under these conditions.

Introduction

One of the most interesting features of the centrosome is that its constituent centrioles duplicate by a semiconservative mechanism. A new (daughter) centriole normally forms only in association with a preexisting paternal (mother) centriole (Vorobjev and Chentsov, 1982; Rieder and Borisy, 1982). Morphologically recognizable new (pro) centrioles appear at the onset of S period (for reviews see Sluder, 1989; Sluder and Rieder, 1996; Hinchcliffe and Sluder, 2001). As a rule, centrosome replication and DNA synthesis are tightly coordinated (Hinchcliffe et al., 1998). This coordination is achieved via a complex regulatory mechanism involving cyclin E(A)-cdk2 activity (Hinchcliffe et al., 1999; for review see Karsenti, 1999; Meraldi et al., 1999), and produces a strict correlation between the number of centrioles and the ploidy of the cell. For example, during G_1, diploid cells contain a 2C amount of DNA and two centrioles, whereas during G_2, cells are 4C and contain four centrioles. This correlation is obviously very important, as the formation of supernumerary centrosomes leads to multipolar mitosis, which, in turn, is thought to cause or contribute to cancerous transformation (Pihan et al., 1998; Carroll et al., 1999; Brinkley, 2001).

Intriguingly, when the centrosome is destroyed by pulses of tightly focused laser light (Khodjakov et al., 2000; Khodjakov and Rieder, 2001) or removed from a cell by micromanipulation (Maniotis and Schliwa, 1991; Hinchcliffe et al., 2001), it does not regenerate. This result is somewhat puzzling, because neither laser ablation nor microsurgery should directly affect genes encoding centrosomal components and already synthesized centrosomal proteins constitutively present in the cytoplasmic pool (Gard et al., 1990; Sluder et al., 1990). Thus, one might expect that the cell still possesses all the components necessary for centrosome assembly but yet, for some reason, it fails to reform.

One potential explanation for the apparent inability of vertebrate somatic cells to regenerate centrosomes is that the centrosome itself contains a specific "template" that provides a unique site and/or pattern for the assembly of the daughter centrioles (for reviews see Fulton, 1971; Marshall and Rosenbaum, 2000). The nature of the hypothetical template is obviously unknown. However, specific precursor structures, such as an annular ring or a fiber, sometimes containing nine distinct "beads" that become nine microtubule triplets, have been observed during basal body (centriole)

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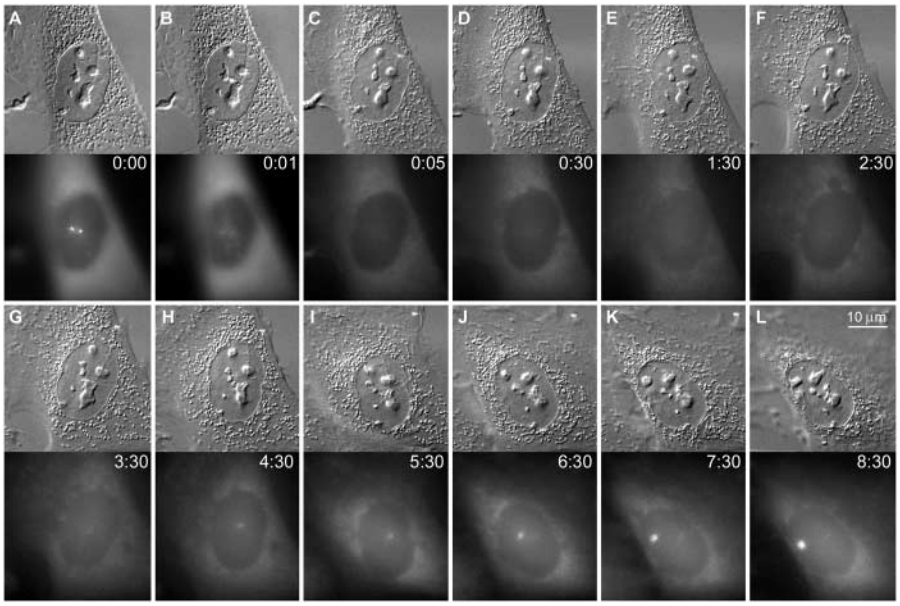

Figure 1. **A new γ-tubulin/GFP focus forms in S-arrested CHO cells after destroying the centrosomes.** Both centrosomes were ablated by laser microbeam (compare A and B), and, after transferring the preparation to a different microscope, the cell was followed by near-simultaneous 3-D fluorescence/DIC time-lapse microscopy (C–L). The top half of each image represents a DIC slice through the central part of the cell, whereas the bottom half is a maximal intensity projection of the entire cell volume recorded at 0.5-μm Z steps. The first sign of a forming γ-tubulin/GFP focus appears ~4–5 h after ablation (H and I). The intensity of this focus then increases rapidly and reaches the level of a typical centrosome between 7 and 8 h after ablation (K and L). Relative to a mature centrosome, the newly formed focus is more diffuse and lacks the typical "subdomain" or hot spot structure (compare A and L). Time in hours:minutes.

duplication (Dippel, 1968; Gould, 1975). It is also possible that the basic template may not even be morphologically recognizable until microtubule triplets are assembled (Sluder et al., 1989). In this regard, when HeLa cells are loaded with antibody against polyglutamylated tubulin (Bobinnec et al., 1998), centrioles disassemble and consequently all centrosomal antigens become dispersed throughout the cytoplasm. However, in sharp contrast to experiments in which the centrosome is removed or completely destroyed (Maniotis and Schliwa, 1991; Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001), as soon as the antibody concentration decreases, centrioles reform and cells eventually regain normal centrosomes (Bobinnec et al., 1998). Again, the difference between centrosome destruction and centrosome dispersion experiments can be explained by the presence of a template associated with the centrosome; destroying the centrosome destroys this template and prohibits a new centrosome from forming, whereas in the dispersion experiments, the template remains (although morphologically undetectable) and later initiates reassembly of the centrosome.

There are, however, examples of centrosome de novo formation in the absence of a preexisting organelle. New centrioles assemble in clam zygotes (Palazzo et al., 1992), mice (Szollosi et al., 1972; Calarco-Gillam et al., 1983), and rabbit blastomeres (Szollosi and Ozil, 1991). This process appears to be peculiar to these systems, because the zygotes of other animals (e.g., sea urchins) normally do not form centrosomes de novo (Sluder et al., 1989). All these examples are in early development, which is driven by large stores of maternal products in the oocyte. The de novo formation of centrioles has also recently been demonstrated in *Chlamydomonas* (Marshall et al., 2001), where the efficiency of this process is ~50% of that normally seen for templated assembly (in association with the maternal centriole). This result, along with the fact that new centrioles normally form only in association with preexisting mother centrioles, implies that in *Chlamydomonas*, the de novo pathway is present but somehow inhibited by an existing centriole (Marshall et al., 2001).

Importantly, the de novo formation of centrioles in *Chlamydomonas* occurs exclusively during the S period of the cell cycle (Marshall et al., 2001). This observation provides an alternative to the template hypothesis for why centrosome de novo formation has not been seen in vertebrate somatic cells. In all of those studies in which the centrosome was removed from the cell, or completely destroyed, progression through the cell cycle was arrested during G_1, before S (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). If one assumes that centrosome de novo formation can occur only during S, then vertebrate somatic cells that lack centrosomes simply never reach that point in the cell cycle where the centrosome can regenerate. In turn, if this assumption is true, then the centrosome should ultimately form de novo in cells lacking centrioles if they are constitutively arrested during S phase.

Here we report that, in fact, when centrosomes are completely ablated by laser microsurgery in CHO cells arrested during S by hydroxyurea (HU)* treatment, centrosomes do form de novo. Initially, new centrosomes consist only of ill-defined pericentriolar material (PCM), but later (~24 h) they also gain centrioles. Unlike during templated formation, the number of centrioles formed de novo in a cell, within a given period, appears to be random. Importantly, the formation of PCM foci is seen to occur even in the absence of microtubules, whereas new centrioles do not form under these same conditions.

Results

Previous centrosome ablation/removal experiments have demonstrated that when cells lacking centrosomes become irreversibly arrested during the G_1 period of the cell cycle

*Abbreviations used in this paper: 3-D, three-dimensional; HU, hydroxyurea; PCM, pericentriolar material.

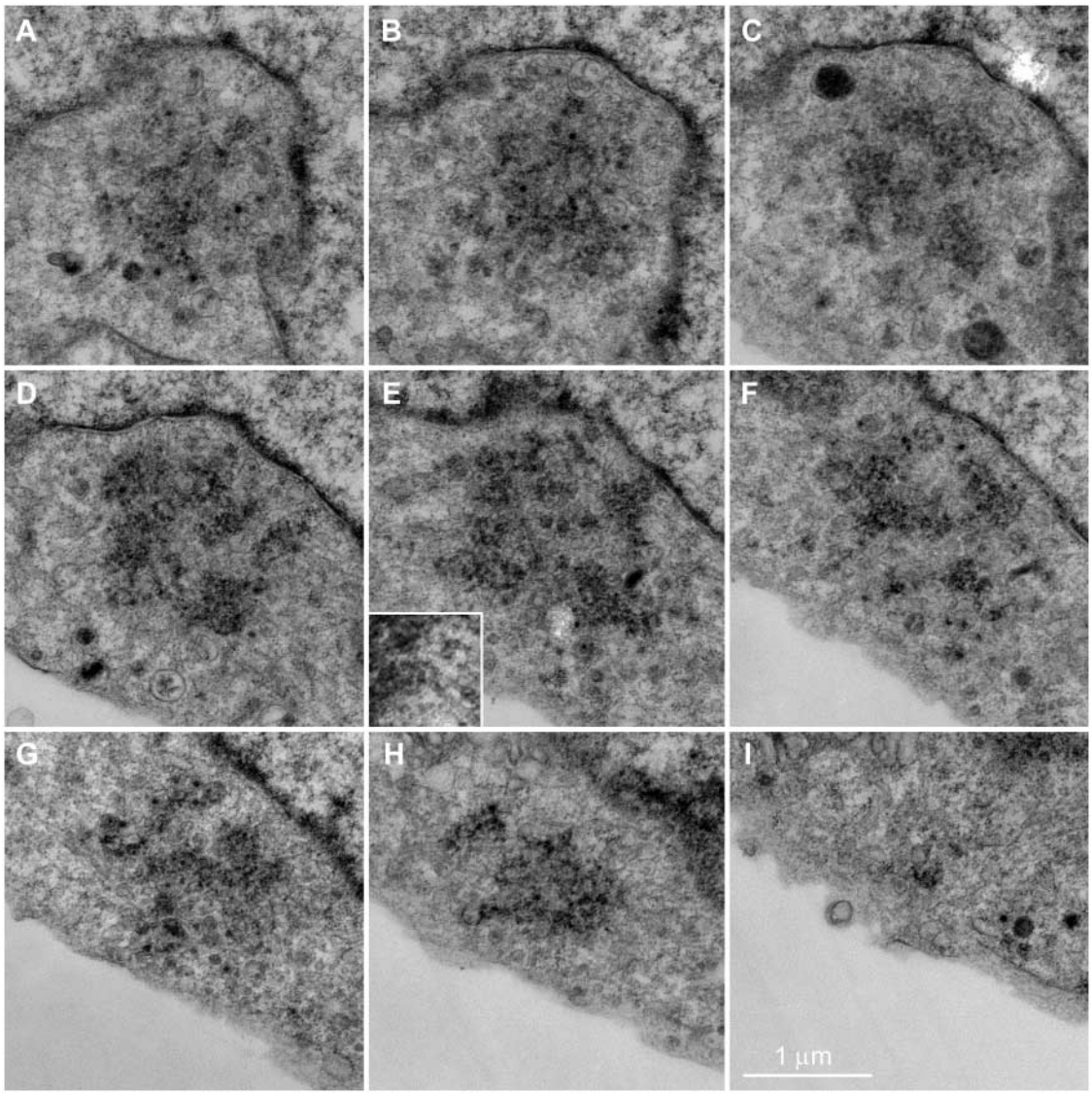

Figure 2. **The γ-tubulin/GFP focus, formed de novo 8–9 h after removing the centrosome, consists of a cloud of PCM, but lacks centrioles.** Serial micrographs from 0.25-μm thick sections through the γ-tubulin/GFP focus from the cell shown in Fig. 1 (matching orientation). The typical electron-dense fiber granular PCM cloud in this cell was found in an invagination of nuclear envelope (A–E) and was roughly spherical with an ~2-μm diameter. Numerous small membrane vesicles closely associated with the PCM (E), and microtubules (E, inset) emanated from the PCM radially.

(Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001), the centrosome does not regenerate for at least several days (Maniotis and Schliwa, 1991; Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). This phenomenon was observed in several different cell types, including CV-1, BSC-1 (both monkey kidney), and PtK_1 (rat kangaroo kidney). We also observed that centrosomes fail to regenerate over a 36-h period when destroyed during G_1 in pig kidney and CHO cells (unpublished data).

To determine if centrosomes can regenerate in cells perpetually arrested in S, we ablated all of the centrosomes in γ-tubulin/GFP-expressing CHO cells that were treated with 2 mM HU. Under this condition, CHO cells have been previously shown to continuously remain in S and to repeatedly replicate their centrosomes (Balczon et al., 1995). To ensure that all cells on the coverslips were already in S during the operation, we pretreated cultures with HU for 18 h, a time equal to the duration of the complete cell cycle, before the ablation.

γ-Tubulin foci reform within 8 h of ablating the centrosome

In all cases (20 experiments) the formation of a new γ-tubulin/GFP focus (foci) was observed after completely destroying the preexisting centrosomes in S-arrested CHO cells. The first signs of a new focus could be detected as early as 4–5 h after the operation. Shortly thereafter, the focus fluorescence intensity and size rapidly increased, reaching parameters typical for a normal centrosome at ~8–10 h after the ablation (Fig. 1). Same-cell correlative serial-section EM revealed that at 8–9 h after the operation, the newly formed γ-tubulin/GFP foci corresponded to extensive clouds of typical electron-dense PCM. These clouds were often located in an invagination of nuclear envelope,

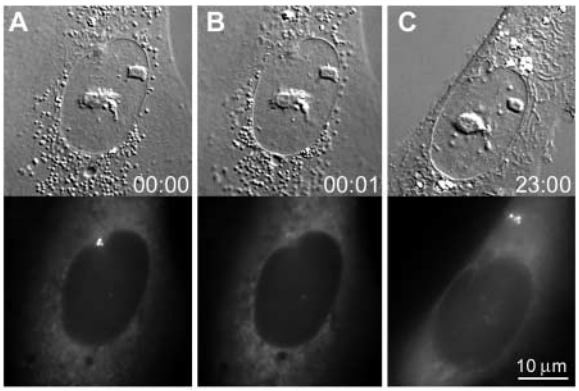

Figure 3. **At later stages (23–24 h), de novo–forming γ-tubulin foci become more compact and structured.** In this example, the centrosome was ablated (compare A and B) and the cell was followed by time-lapse microscopy for 23 h (see Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200205102/DC1). Note that contrary to earlier stages (Fig. 1 L), by 23 h, the structure of the de novo–formed focus is very similar to that of a typical centrosome (compare A and C). Panel layout as in Fig. 1.

and were associated with a large number of small vesicles and Golgi cisternae (Fig. 2). Numerous microtubules emanated from the PCM (Fig. 2 E, inset). In all six cells reconstructed by serial-section EM, newly formed PCM foci lacked centrioles or any identifiable remnants/precursors of centrioles.

New centrioles appear 24 h after ablating the centrosome

To determine the structure of the de novo–formed PCM foci at later times, we followed cells in which the centrosome was completely ablated for ~24 h. For these experiments, cells were transferred immediately after the operation to a low-power phase-contrast microscope and imaged every 15 min (see supplemental videos, available at http://www.jcb.org/cgi/content/full/jcb.200205102/DC1). After 24 h, the cells were transferred back to a high-resolution microscope where three-dimensional (3-D) γ-tubulin/GFP fluorescence images were collected. After collecting these images, the cells were then fixed and processed for either an immunocharacterization of the centrosome and microtubule pattern or for correlative serial-section EM.

At the light microscopy level, the γ-tubulin/GFP foci formed de novo were indistinguishable from normal centrosomes (Fig. 3). In contrast to the 8-h time point, when each focus appeared as a single relatively amorphous cloud, by 24 h, all foci (12 cells) exhibited a more compact organization: each contained clearly defined "hot spots" embedded within a more amorphous and fluorescently less intense γ-tubulin/GFP cloud (Fig. 3; Fig. 4 I; Fig. 6). This distribution of γ-tubulin is typical for normal centrosomes in which PCM is usually organized around centrioles.

Serial-section EM revealed that the γ-tubulin/GFP hot spots generally correlated with compact pieces of PCM, many of which contained associated clusters of virus-like particles characteristic of the centrosome in CHO cells (Gould and Borisy, 1977). Numerous microtubules emanated from the PCM, and multiple Golgi cisternae were usually found close to the PCM bodies (Fig. 4). The distribution of the PCM bodies generally corresponded to the hot spots within the γ-tubulin/GFP pattern (Fig. 4; also see Fig. 9). There were, however, occasional patches of PCM that did not correspond to a major hot spot of γ-tubulin/GFP density (Fig. 4). At this time, some of the PCM patches contained centrioles (Fig. 4).

The number of centrioles per cell ranged from 2 to 14 (in five serially reconstructed cells). Most of the centrioles exhibited a normal morphology but some were obviously aberrant. The types of abnormalities included partially open centriolar cylinders, distorted/bent walls, and different cylinder lengths (Fig. 5). These abnormalities were very similar to those we found during centriole reassembly after loading HeLa cells with antipolyglutamylated tubulin antibody (Bobinnec et al., 1998).

Although all cells analyzed contained multiple centrioles, they always formed a common complex, which is not unusual for CHO cells (Balczon et al., 1995). This complex was often positioned in close proximity of the nuclear envelope. However in some cells (Fig. 3), the centrosome could be well separated from the nuclear membrane and off center in the cell.

Newly formed γ-tubulin foci also contain other centrosomal proteins and organize multipolar spindles during mitosis

Immunostaining revealed that newly formed γ-tubulin foci also contained pericentrin and ninein, two well-characterized bona fide centrosomal components. The pericentrin distribution was largely identical to that of γ-tubulin (unpublished data). Ninein was concentrated only in parts of the volume occupied by γ-tubulin. The brightest spots in the ninein pattern were usually seen immediately adjacent to the brightest spots in the γ-tubulin pattern (Fig. 6). This distribution matches well the distribution of ninein in control centrosomes, where this protein is largely concentrated in the centriolar appendages (Mogensen, 1999; Piel et al., 2000). We did not, however, observe morphologically well-defined appendages on any of the centrioles formed de novo 24 h after centrosome ablation.

Because newly formed γ-tubulin foci are also enriched in at least two other integral centrosomal components, and often contain centrioles, we conclude that a complete centrosome forms de novo in S-arrested CHO cells. That these centrosomes are functional as microtubule-organizing centers is evident from the fact that numerous microtubules are seen to emanate from them in our EM and light microscopy preparations (Figs. 2 and 6), and a typical radial microtubule repolymerization pattern is seen in experimental cells treated and then released from a 1-h nocodazole block (unpublished data). The question remained, however, whether these centrosomes can serve as proper polar organizers once the HU is washed out. To answer this question, we ablated the original centrosome and let a new one form in the presence of HU. Then, 24 h after ablating the original centrosome, the cells were released from HU into growth medium containing 10 mM caffeine to induce rapid entry into mitosis in HU-arrested CHO cells (Balczon et al., 1995; Wise and Brinkley, 1997).

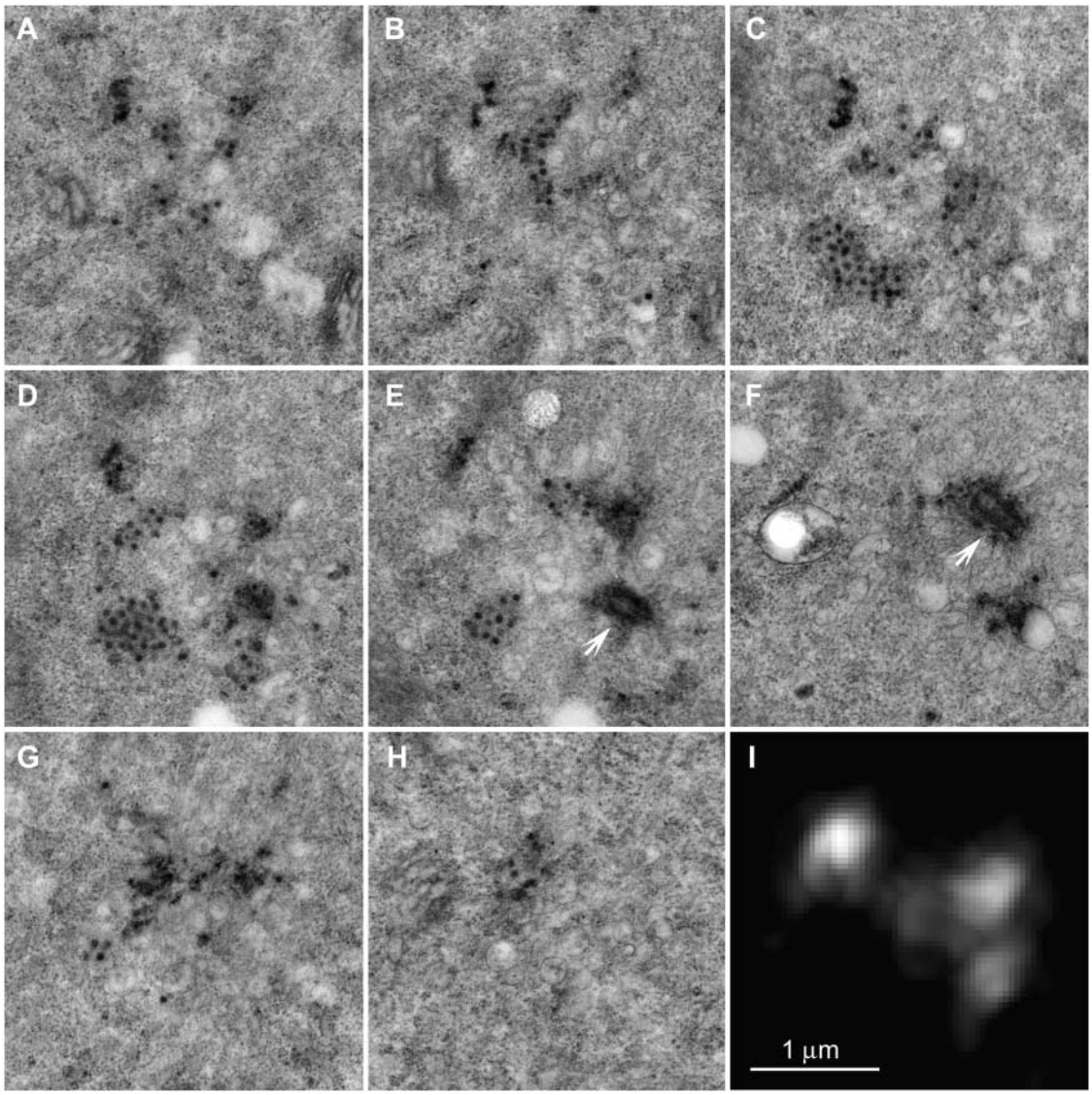

Figure 4. **Centrioles form de novo within γ-tubulin foci ~24 h after destroying the centrosome.** (A–H) Serial micrographs of sequential 0.25-μm sections through the γ-tubulin focus shown in Fig. 3, as well as (I) the maximal intensity projection of the γ-tubulin/GFP fluorescence restored on a subpixel grid (55-nm pixels). Note that both centrioles (E and F, arrows) are associated with PCM clouds that correspond to γ-tubulin–enriched areas (I). There are, however, clouds of PCM highly enriched in γ-tubulin that do not contain centrioles (compare B and C and I) as well as clouds of PCM that appear to contain lesser amounts of γ-tubulin (compare C and D and I).

Under these conditions, 9 out of 13 cells with de novo–formed centrosomes underwent multipolar mitosis within 2–10 h after the addition of caffeine. During mitosis, the cells rounded to the point where it was not possible to determine exact architecture of the spindle during metaphase. However, in eight of the nine cells, multiple cytokinesis furrows formed during telophase (Fig. 7). Because many of these later regressed, only three (two cases) or two (three cases) daughter cells were ultimately formed. In three cases, all furrows ultimately failed, resulting in the formation of just one daughter cell. Importantly, however, regardless of the cleavage pattern, all daughter cells contained multiple nuclei. This feature clearly indicates that in all of these cells the spindles were multipolar. In the remaining cell, only one furrow formed and two mononuclear daughter cells were produced, indicating that in this cell the spindle was bipolar (unpublished data). From these observations, we conclude that the great majority of the cells containing de novo–formed centrosomes assemble a multipolar spindle during mitosis.

γ-Tubulin foci, but not centrioles, form in the absence of microtubules

To determine if de novo centrosome formation depends on the presence of a microtubule network, we arrested CHO cells in S with HU, and then completely depolymerized their microtubules with 5 μM nocodazole. Nocodazole was added to the media ~1 h before laser ablation and was continuously present for the subsequent 24-h period. Under this condition, CHO cells lack cytoplasmic microtubules (unpublished data; Balczon et al., 1999).

In all 10 cells examined, γ-tubulin foci formed de novo in the absence of cytoplasmic microtubules, and at about the

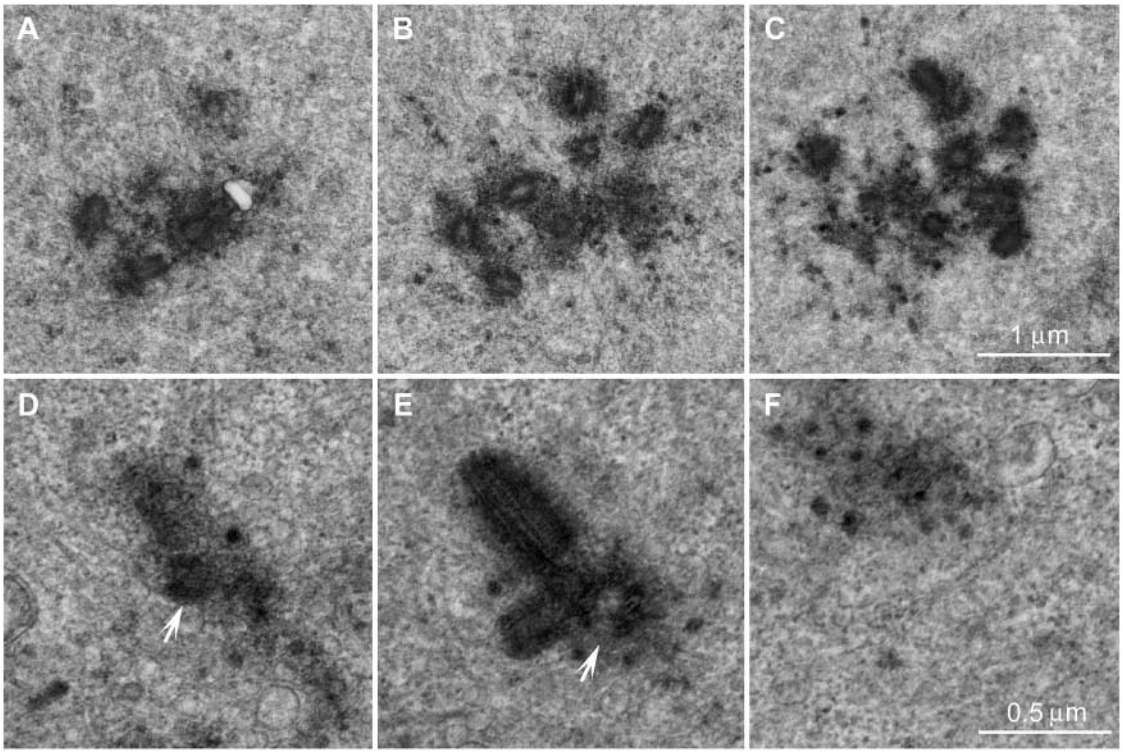

Figure 5. **γ-Tubulin foci formed de novo contain variable numbers of centrioles 24 h after removing the original centrosome.** Micrographs of sequential sections through two different de novo–formed γ-tubulin foci. (A–C) A cell that contained 14 centrioles, many of which are structurally defective. (D–F) Complete series from a different cell at a higher magnification illustrating several centriolar defects, including very short blades (D, arrow) and open cylinders missing some microtubule triplets (E, arrow).

same time as in cells with intact microtubules (see above). A striking difference was that in most nocodazole-treated cells (7 out of 10), several γ-tubulin/GFP spots formed instead of a single larger common complex (Fig. 8).

Correlative serial-section EM of three cells fixed 24 h after the centrosome ablation revealed that the γ-tubulin/GFP foci formed in nocodazole-treated cells, corresponding to PCM bodies that were indistinguishable from those formed in the presence of microtubules (Fig. 9). The only obvious difference was that these cells consistently lacked centrioles. Immunostaining revealed that the γ-tubulin foci formed in nocodazole-treated cells were also enriched in pericentrin and ninein (unpublished data).

Discussion

The semiconservative mechanism for centrosome replication has fascinated biologists for generations. Some cell types are known to contain enough centrosomal subunits to assemble numerous centrosomes (Gard et al., 1990; Sluder et al., 1990), and yet during each individual cell cycle only one new centrosome is assembled, and it is constructed in close spatial association with the preexisting organelle. The question remains as to why cells, known to contain an ample supply of centrosomal subunits, do not spontaneously form multiple centrosomes. In the past, the most popular explanation for this was that the preexisting centrosome contains

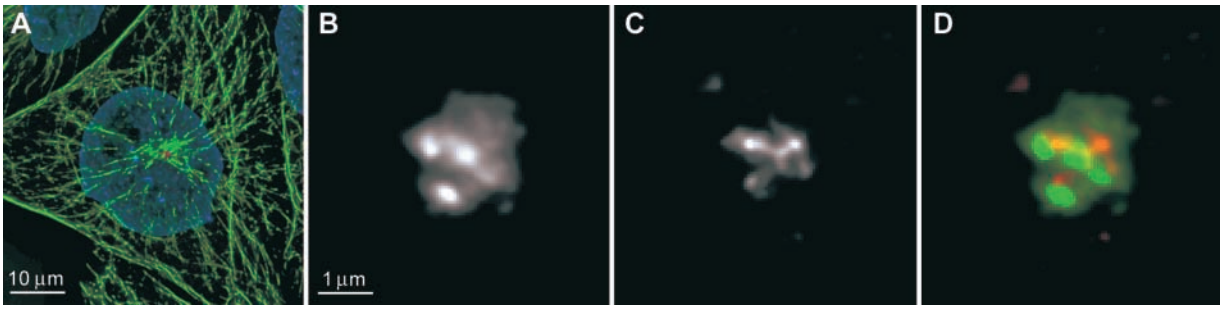

Figure 6. **Centrosomes formed de novo organize radial arrays of microtubules and contain ninein that is normally associated with the mature (mother) centriole.** (A) Maximal-intensity projection representing microtubules (α-tubulin staining in green), centrosome (γ-tubulin staining in red), and the nucleus (Hoechst 33342 in blue). The impression of discontinuity in some of the microtubules arises from projecting too many individual optical sections on one plane, which was necessary because the centrosome is positioned on the dorsal surface of the nucleus. (B) γ-Tubulin; (C) ninein; (D) B and C merged and pseudocolored.

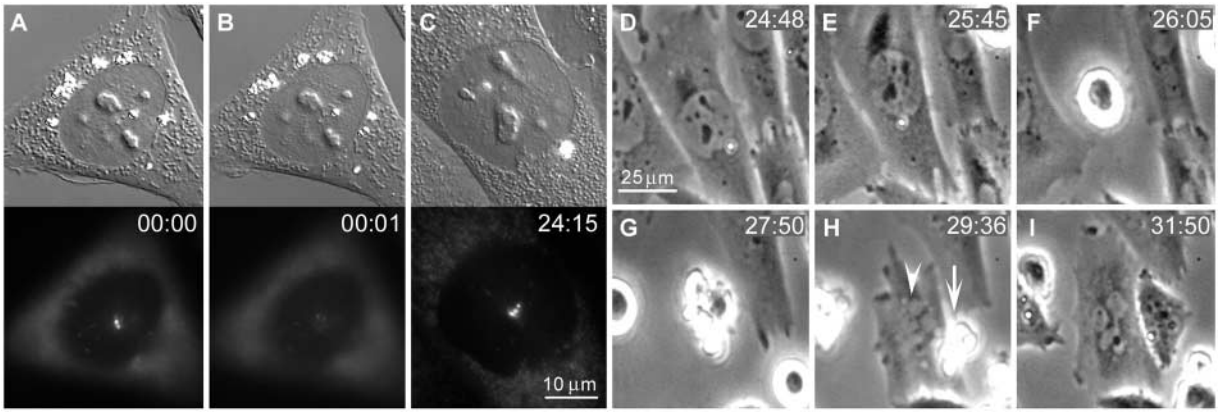

Figure 7. **Mitosis in cells with de novo–formed centrosomes.** In this example, the centrosome was ablated (compare A and B) and the cell was followed by time-lapse microscopy for 24 h (see Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200205102/DC1). During this time a new centrosome was formed (C). At 24:20 after the operation, the cell was stimulated to enter mitosis by adding 10 mM caffeine. Approximately 1.5 h later, the cell began condensing its chromosomes (E) and then entered mitosis (F). 2 h later, the cell formed multiple cytokinesis furrows (G), demonstrating that the spindle formed in this cell was multipolar. All but one of these cytokinesis furrows ultimately regressed and only two unequally sized daughter cells were formed (H). Both of these cells contained multiple nuclei (I), which is additional evidence that the mitotic spindle formed in this cell was multipolar. Panel layouts in A and B as in Fig. 1. (D and E) Phase contrast. Time in hours:minutes.

a template that is essential for somehow initiating the formation of a new centriole (for reviews see Fulton, 1971; Marshall and Rosenbaum, 2000). Although the nature of this hypothetical template has never been defined, it has been speculated that centrioles contain specific genetic information in the form of RNA or DNA (Hall et al., 1989). Most of the reports leading to this idea have, however, been disproven (Johnson and Rosenbaum, 1990).

There are somatic cells that are capable of forming numerous centrosomes during a single interphase. For example, during ciliogenesis, some epithelia generate hundreds of basal bodies (Dirksen, 1991). Here, new centrioles (basal bodies) appear to form in association with ill-defined fibrogranular bodies called the deuterosomes (Anderson and Brenner, 1971), and the formation of deuterosomes always precedes that of the basal bodies. Even though the deuterosome has little structural resemblance to the basal body/centriole, it still appears to act as a template for centriole assembly. Moreover, because the formation of multiple cilia occurs in cells that possess a preexisting centrosome, it is possible that this centrosome templates the formation of deuterosomes. If true, this would imply that the basal bodies in ciliated epithelial cells are formed via the same templated replication mechanism as normal centrioles.

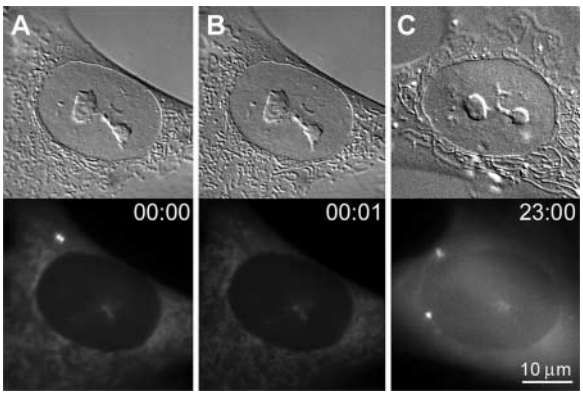

Figure 8. **Formation of new γ-tubulin/GFP foci in S-arrested CHO cells pretreated with 5 μM nocodazole.** The single centrosome was completely ablated (compare A and B) and the cell followed by time-lapse microscopy (see Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200205102/DC1). Note that contrary to centrosome formation in control cells, in the absence of microtubules, several (in this case, two) widely separated γ-tubulin/GFP foci are formed 23 h after destroying the original centrosome. Panel layout as in Fig. 1.

We are aware of only one study that claims to document the true de novo formation of centrosomes in vertebrate somatic cells. Zorn et al. (1979) reported that centrioles regenerate in karyoplasts obtained by enucleating L929 cells with centrifugation in the presence of cytochalasin B. A statistical EM analysis of random sections from cell pellets led these authors to conclude that centrosome regeneration occurs with a very low frequency, and that cells do not undergo mitosis before they regenerate a complete set of centrioles. Although provocative, this conclusion has always been questioned because it was based on a method considered unreliable by modern standards.

Our finding that centrosomes reform in cells constitutively arrested during S by HU reveals, for the first time, that new centrosomes can and do assemble in vertebrate somatic cells in the absence of a preexisting centrosome (de novo). The de novo formation is not limited to only CHO cells. We have also observed the formation of multiple centrioles de novo in HeLa and hTERT cells (unpublished data). Surprisingly, the de novo formation of the centrosome is very efficient, as evident from the fact that all cells in our experiments ultimately reformed a centrosome. This unexpected efficiency raises the question of whether we completely destroyed the centrosome in our experiments, or if it was only damaged and regrew from the remnant. Several observations reveal that our approach completely eliminates the centrosome. First, ablating this organelle by laser microsurgery abolishes its microtubule-

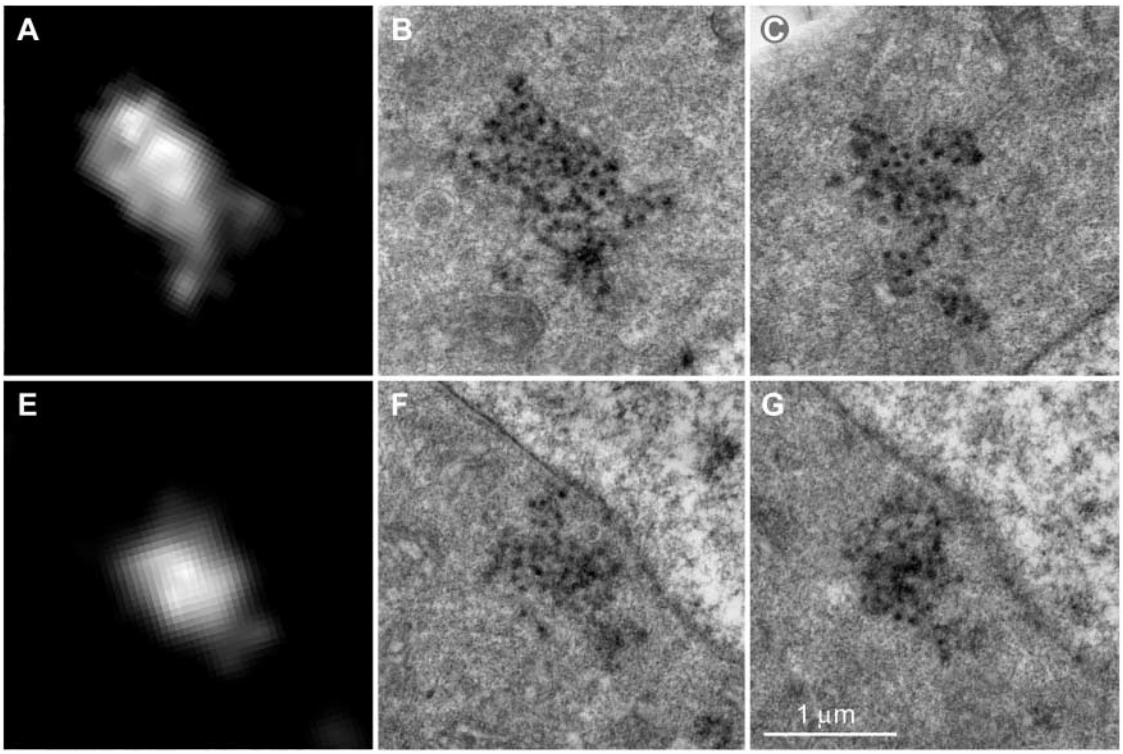

Figure 9. **γ-Tubulin foci formed in the absence of microtubules contain well-defined clouds of PCM but lack centrioles.** Maximal-intensity projection (A and E) of γ-tubulin/GFP fluorescence, restored on a subpixel grid (55-nm pixels), of the foci shown in Fig. 8. Panels B and C and F and G are corresponding serial images of 0.25-μm sections through each focus. Note that each contains well-defined PCM clouds but no centrioles.

nucleating potential (Khodjakov et al., 2000; Khodjakov and Rieder, 2001). Second, we never observed regeneration of the centrosome, defined by the formation of a γ-tubulin/GFP focus and by the accumulation of PCM at the electron microscopic level, when it was destroyed in cells that were not arrested in S (present study; Khodjakov and Rieder, 2001). Third, all of the cells that were reconstructed by serial-section EM 8–9 h after destroying the centrosome lacked centrioles. This would only be expected to occur if the preexisting centrosome was completely destroyed by the laser microsurgery. We can also rule out a possibility that a piece of PCM, containing γ-tubulin, survived the operation and was not apparent because it was simply photobleached; centrosome-associated γ-tubulin is in constant dynamic exchange and a photobleached centrosome recovers >50% of its original intensity in ~1 h (Khodjakov and Rieder, 1999). However, we never observed the formation of new γ-tubulin foci until ~5 h after the operation. Fourth, the number of centrioles found in cells 24 h after ablation was highly variable, reaching up to 14 centrioles/cell. Considering that the number of centrioles in HU-arrested cells doubles approximately every 20 h (Balczon et al., 1995), it is not possible to produce 14 centrioles in 24 h by the templated assembly unless the cell contained seven centrioles at the completion of our laser ablation. There is no possibility that we would not detect seven centrioles in an individual cell by our GFP/imaging approach. Finally, we always selected cells in which all preexisting centrosomes were in one complex at the moment of the operation (the great majority of CHO cells). Yet, in these cells, new γ-tubulin foci formed in various regions of the cytoplasm in the absence of microtubules. Because centrosomes are not motile without microtubules (Khodjakov and Rieder, 1999), at least some of the new centrosomes must have formed in the area of cytoplasm distant from the site of the original centrosome.

Although the molecular mechanism of de novo centrosome formation remains to be elucidated, our experiments reveal several important features of this process. First, the process of de novo formation takes ~24 h, which is greater than the duration of a complete cell cycle in CHO cells. This timing offers a straightforward explanation for why centrosomes do not regenerate in cells lacking centrosomes, when they are not delayed in S (Hinchcliffe et al., 2001); cells normally spend less time in S than the time required to form a centrosome by the de novo formation. It is also evident from our previous studies that de novo centrosome formation does not occur in G_1 (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). Although we have not directly tested whether the de novo formation can occur during G_2, it has been shown that centrioles do not replicate in cells arrested in G_2 for >20 h (Balczon et al., 1995). These data suggest that de novo assembly of the centrosome requires specific cytoplasmic conditions, such as high activity of cdk2/cyclin A/E. In this regard it has been shown that similar conditions are also required for templated centriole replication (for review see Hinchcliffe and Sluder, 2001). The second important feature is that the very first signs of the centrosome de novo assembly, the formation of a γ-tubulin–enriched focus, can be detected only 4–5 h after ablation. This delay could indicate a need for a specific gene expression and/or protein synthesis. Although identifying

putative genes and proteins involved in the process of centrosome de novo formation is beyond the scope of our current study, preliminary data indicate that the de novo pathway is only activated after the last centriole is ablated (unpublished data). Thus, if gene expression is required for the de novo formation, the genes involved might not be expressed if the cell contains a single centriole.

Our demonstration that the centrosomes formed de novo contain a random number of centrioles, 24 h after ablating the original centrosome, also reveals that the de novo pathway supports the parallel production of multiple centrioles. This condition would have catastrophic consequences for the cell, as redundant centrosomes lead to the formation of multipolar spindles, which in turn produce aneuploid daughter cells (for review see Brinkley, 2001). Fortunately, the de novo assembly of centrosomes in vertebrate cells appears to be somehow inhibited in the presence of a centrosome. This is evident from the fact that the number of centrioles in HU-arrested CHO cells containing centrosomes increases gradually, doubling every 20 h, as would be expected for the templated mechanism (Balczon et al., 1995). If the de novo formation was to occur in parallel to the templated assembly under these conditions, the number of centrioles should have increased much faster. In this respect, an inhibitory mechanism by which existing centrioles suppress the de novo assembly pathway may also exist in *Chlamydomonas* (Marshall et al., 2001). Together, these studies imply that the template mechanism for centriole replication is needed, not because the de novo pathway is inefficient, but rather because it sets limits on the number of centrioles produced during each individual cell cycle (Hinchcliffe and Sluder, 2001).

Our data reveal that the de novo formation of centrosomes occurs in two steps. First, a loose cloud of electron-opaque material containing centrosomal proteins (PCM), including γ-tubulin and pericentrin, forms in the cytoplasm. This cloud is capable of organizing microtubules into a typical radial pattern. Interestingly, the formation of such well-defined clouds of the PCM occurs even in the absence of microtubules. However, unlike in cells containing microtubules, which form a single relatively large PCM focus, in the absence of microtubules, several individual foci of PCM form within the cytoplasm. This pattern would be expected if smaller pieces of PCM are initially assembled independently in different parts of the cell and then delivered to a common location via microtubule-based transport. This mechanism of centrosome formation is consistent both with a microtubule-independent recruitment and an exchange of individual centrosomal components (Stearns and Kirschner, 1994; Moritz et al., 1998; Schnackenberg et al., 1998; Khodjakov and Rieder, 1999), as well as a microtubule-dependent transport of relatively large preassembled pieces of PCM toward the centrosome (Young et al., 2000; also see Dictenberg et al., 1998; Balczon et al., 1999).

Over time, the forming cloud of PCM becomes more compact and better structured, and this correlates with the appearance of new centrioles (~24 h). These changes in the structure of the PCM are consistent with the idea that centrioles function as spatial organizers for the PCM, as proposed by Bobinnec et al. (1998). In this study, centrioles were disrupted by microinjection of an anticentriolar antibody, after which all PCM components were scattered. Then, as the antibody concentration decreased below a threshold level, centrioles reappeared and PCM once again became focused. In their original interpretation, Bobinnec et al. (1998) favored the idea that centriole reformation in this system occurs via morphologically unidentifiable "centriolar organizers" that remain in the cell. However, several similarities between the reformation of centrioles in the Bobinnec et al. (1998) paper and our data, reported here, suggest that the observations of Bobinnec et al. (1998) can also be explained by de novo assembly. First, the reappearance of centrioles after antibody microinjection occurs gradually, and at intermediate time points, most cells contained incomplete centrioles that were remarkably similar to those we observed during the de novo formation. Second, many of the mitotic cells reconstructed 60 h after loading with the antibody contained more than the expected number of fully formed or partial centriolar cylinders (see Fig. 10 in Bobinnec et al., 1998). This can be easily explained because, as argued above, the de novo pathway supports parallel formation of multiple centriolar cylinders. If the phenomenon observed in the Bobinnec et al. (1998) study does represent a true de novo formation of the centrosome, it has very important ramifications. Because it appears occur in cells that are not arrested in S, the centrosome de novo formation pathway would be unregulated in highly transformed cells, like HeLa. In this respect, it is worth noting that HeLa and some other cell types are reported to initiate centriole replication prematurely during G_1 (Phillips and Rattner, 1976; Fukasawa et al., 1996). Currently, we are evaluating the possibility that these types of cells can form centrosomes de novo during G_1.

Materials and methods

Cell culture

A stable clone, constitutively expressing γ-tubulin/GFP, was isolated from the CHO-KK parental cell line (Burki et al., 1980) by G-418 selection and limited-dilution cloning. These cells express growth characteristics very similar to the parental line. Cells were maintained in Ham's F12 media supplemented with 10% FBS at 37°C in humidified atmosphere with 5% CO_2.

For laser microsurgery, cells were transferred onto 24 × 24-mm coverslips in 50-mm Petri dishes. 18 h before the experiment, HU (Sigma-Aldrich) was added to a 2-mM final concentration. Approximately 30 min before each experiment, the cultures were mounted in Rose chambers in phenol-free L15 media, supplemented with 10% FBS, and 2 mM HU. For some experiments, 5 μM nocodazole (Sigma-Aldrich) was also added to the media at this time. During laser microsurgery, cells were kept at 34–37°C by a Rose chamber heater (Rieder and Cole, 1998).

Laser microsurgery

Centrosome ablation by laser microsurgery has been previously detailed (Khodjakov et al., 2000; Khodjakov and Rieder, 2001). In brief, pulses of 532-nm Nd:YAG laser light are focused by a 60X 1.4 NA lens so the effective waist of the beam is ~0.5 μm in the specimen plane. Centrosomes are then destroyed by exposing them to the light pulses until the γ-tubulin/GFP fluorescence is completely abolished. This typically takes ~10 s and requires two to three series of 20–30 laser pulses.

Images were captured by a MicroMax 5 MHz cooled CCD camera (Princeton Instruments) and saved as 8-bit TIFF files. The imaging system is driven by Image Pro software (Media Cybernetic).

Long-term imaging

After laser microsurgery, the position of the experimental cell was marked on the coverslip, and the culture was transferred to a phase-contrast microscope equipped with a Rose chamber heater (Rieder and Cole, 1998).

Time-lapse images were then captured every 15 min for 24 h using a video-rate CCD camera (Model 100; Paultek Imaging). Illumination was obtained from a 100-W tungsten filament, filtered to remove UV (GG400) and IR (KG5) components, made monochromatic (GIF 546), and shuttered (UniBlitz Electronics) between exposures (~2 s/image).

EM

Cells followed in vitro were fixed and prepared for EM according to standard protocols (Khodjakov et al., 1997; Rieder and Cassels, 1999). After flat embedding, they were relocated using phase-contrast microscopy and then serially thick sectioned (0.25 μm). The sections were then imaged and photographed in a ZEISS 910 microscope operated at 100 kV.

Immunofluorescence microscopy and deconvolution

For immunofluorescence analysis, cells, previously followed by time-lapse microscopy, were permeabilized with 1% Triton X-100 in PEM buffer (100 mM Pipes, 1 mM EGTA, 5 mM Mg^{2+}, pH 6.9) for 1 min and fixed with 1% glutaraldehyde in PEM. The following antibodies were used: monoclonal anti–γ-tubulin (T6557; Sigma-Aldrich) at 1:300; monoclonal anti-ninein (gift of Dr. M. Bornens, Institut Curie, Paris, France) at 1:200; polyclonal anti-pericentrin (gift of Dr. S. Doxsey, University of Massachusetts, Worcester, MA) at 1:200; and anti–α-tubulin (YL1/2; gift of Dr. J. Kilmartin, Medical Research Council, Cambridge, UK) at 1:100.

Immunofluorescence images were collected as 3-D volumes on a Nikon Eclipse TE-200 microscope equipped with filter wheels (LEP), a piezo Z positioner (Physik Instrumente), and a Nikon 60XA 1.4 NA PlanApo lens. Some of the data sets were subsequently restored on a subpixel grid and deconvolved using blind deconvolution algorithms developed at AutoQuant Imaging, Inc.

Online supplemental material

Time-lapse movies (supplemental videos 1–3) of the cells presented in Figs. 3, 7, and 8 are available at http://www.jcb.org/cgi/content/full/jcb.200205102/DC1.

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