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Sabrina La’Terra  
New York State Department of Health

Christopher N. English  
University of Massachusetts Medical School

Poll J. Hergert  
New York State Department of Health

See next page for additional authors

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Authors
Sabrina La Terra, Christopher N. English, Polla J. Hergert, Bruce F. McEwen, Greenfield Sluder, and Alexey Khodjakov

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The de novo centriole assembly pathway in HeLa cells: cell cycle progression and centriole assembly/maturation

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It has been reported that nontransformed mammalian cells become arrested during G1 in the absence of centrioles (Hinchcliffe, E., F. Miller, M. Cham, A. Khodjakov, and G. Sluder. 2001. Science. 291:1547–1550). Here, we show that removal of resident centrioles (by laser ablation or needle microsurgery) does not impede cell cycle progression in HeLa cells. HeLa cells born without centrosomes, later, assemble a variable number of centrioles de novo. Centriole assembly begins with the formation of small centrin aggregates that appear during the S phase. These, initially amorphous “precentrioles” become morphologically recognizable centrioles before mitosis. De novo–assembled centrioles mature (i.e., gain abilities to organize microtubules and replicate) in the next cell cycle. This maturation is not simply a time-dependent phenomenon, because de novo–formed centrioles do not mature if they are assembled in S phase–arrested cells. By selectively ablating only one centriole at a time, we find that the presence of a single centriole inhibits the assembly of additional centrioles, indicating that centrioles have an activity that suppresses the de novo pathway.

Introduction

The centrosome in somatic cells consists of two centrioles surrounded by a cloud of amorphous pericentriolar material (PCM) containing the γ-tubulin ring complexes involved in microtubule nucleation (Moritz et al., 1995; Zheng et al., 1995; Schnackenberg et al., 1998). Because the PCM accumulates around the centrioles (Bobinnec et al., 1998), the number of centrioles ultimately defines the number of functionally active centrosomes in the cell (Rieder et al., 2001).

New or “daughter” centrioles normally form in association with preexisting “mother” centrioles approximately at the G1/S transition. The mother and the daughter remain physically linked until after the ensuing mitosis (Vorobjev and Chentsov, 1982; Rieder and Borisy, 1982). As a result, each daughter cell inherits one centriole that is <1 cell cycle old and one that was formed ≥1.5 cell cycles ago. The assembly of just a single new centriole in close spatial association with the mother indicates that centriole formation normally requires a special site for its assembly, which is present only in the proximal end of a mature centriole. This ensures that centriole duplication is under tight numerical and spatial control. Failure of the cell to precisely regulate centrosome number leads to multipolar mitotic spindles and unequal chromosome distribution at mitosis. This, in turn, results in aneuploidy, genetic imbalances, and genomic instability that are hallmarks of aggressive human tumors (for reviews see Brinkley, 2001; Salisbury et al., 1999).

Under certain circumstances, centrioles can also assemble de novo. This process has been reported in a number of cell types, including lower eukaryotes (Marshall et al., 2001; Suh et al., 2002), oocytes (Palazzo et al., 1992), blastomers (Szollosi et al., 1972), and parthenogenetically developing embryos (Kallenbach and Mazia, 1982; Szollosi and Ozil, 1991; Riparbelli and Callaini, 2003). The de novo assembly has also been observed in vertebrate somatic cells, albeit only in CHO cells artificially arrested in S phase (Khodjakov et al., 2002). In this system, the de novo pathway results in simultaneous formation of a variable number of centrosomes over 24 h, which is the time comparable with the duration of the cell cycle in these cells.

The practical significance of the de novo pathway remained unclear, because normal vertebrate somatic cells, from which the centrosome has been removed, arrest in G1 and do not form centrosomes de novo (Maniotis and Schliwa, 1991; Hinchcliffe...
et al., 2001; Khodjakov and Rieder, 2001). Thus, to further advance our understanding of de novo centrosome formation in mammalian cells, we sought to test if this phenomenon is peculiar to CHO cells arrested in S phase. We turned to HeLa cells because their cell cycle may be less strictly linked to the presence of the centrosome than is in other cell types. HeLa cells can progress through the cell cycle when the centrosome is dispersed by microinjection of antipolyglutamylated tubulin antibody (Bobinnec et al., 1998). Using HeLa cells stably transfected with centrin-1/GFP to mark centrioles (Piel et al., 2000), we ablated one of the centrosomes at metaphase. Upon the completion of mitosis, such cells divided into a centrosome-containing and an acentrosomal daughter cells. Contrary to what was found when centrosomes were removed from untransformed BSC1 and CV1 cells (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001), we observed that acentrosomal HeLa cells continue to progress through the cell cycle and reform centrioles de novo.

**Results**

**HeLa cells born without centrosomes progress through the cell cycle and form centrioles de novo**

All experiments were conducted in HeLa cells stably transfected with human centrin-1/GFP. This protein accumulates inside the centriole lumen from the earliest stages of centriole formation, which makes it a reliable marker for individual centrioles (Piel et al., 2000). Using the GFP signal as a target, we laser ablated both centrioles (diplosome) associated with one of the two spindle poles during mitosis. At the completion of mitosis, this produces two genetically identical daughter cells: one with and one without a centrosome. Comparative analysis of the two sisters allowed us to differentiate between the effects specific to the centrosome ablation versus potential nonspecific effects of laser irradiation and long-term fluorescence imaging. It is important to emphasize that the laser beam destroys all structures/proteins within an \( \sim 0.4-0.6 \mu m \times \text{x-y-z} \) volume. When we aimed the laser at a centriole, the beam destroyed both the centriole and the PCM associated with the targeted centriole. Thus, ablation of both centrioles results in the structural and functional destruction of the entire centrosome (Khodjakov et al., 1997, 2000; for review see Khodjakov and Rieder, 2004). However, it is possible to ablate just a single centriole (along with its associated PCM) within a diplosome so that the other centriole (and its PCM) remain intact (see Materials and methods).

We found that complete ablation of one of the two centrosomes in HeLa cells at metaphase or early anaphase did not affect cytokinesis, reconstitution of nuclei, and postmitotic flattening (Fig. 1). Time lapse microscopy revealed that the centrosomal and acentrosomal sisters are morphologically indistinguishable from each other and exhibit similar behavior, which is consistent with our previous observations in CV-1 and PtK\(_1\) cells (Khodjakov et al., 2000; Khodjakov and Rieder, 2001). However, in sharp contrast with nontransformed cells (e.g., CV-1, BSC-1, or PtK\(_1\)) that arrest during G\(_1\) in the absence of centrosomes (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001), HeLa cells born without centrosomes progressed through the cell cycle with normal timing. These cells entered the next mitosis at 31.9 \( \pm \) 7.1 h (\( n = 16 \)) versus 30.6 \( \pm \) 7.3 h (\( n = 14 \)) for their centrosomal sisters. To standardize our descriptions of the progression through several consecutive cell cycles for these cells, we will hereafter refer to the completion of the mitosis during which the centrioles were ablated as the “birth” of the cell. After birth, the cells underwent a “first” cell cycle that culminates in the “first mitosis”, and then the “second cell cycle” and “second mitosis,” etc.

Approximately 20–25 h into the first cell cycle, a number of minuscule aggregates of centrin/GFP appeared in the cytoplasm of cells that were born without centrioles. Initially, these aggregates were barely recognizable against the diffuse centrin/GFP background fluorescence (Videos 1 and 2, available at http://www.jcb.org/cgi/content/full/jcb200411126/DC1). Their intensity gradually increased until they reached the levels typical for normal centrosomes in this cell line (Fig. 2 and Videos 1 and 2). The increase in intensity was usually completed just before or during first mitosis (30–35 h after the cell’s birth). The number of aggregates was variable (from 2 to >10 per cell); however, once the initial aggregates became detectable, their number in an individual cell did not increase over time.
This indicated that the formation of the aggregates in each cell occurred within a relatively short period of time instead of gradually accumulating as the cell progressed through the cell cycle.

Correlative GFP fluorescence light microscopy/serial-section EM analyses revealed that these aggregates are amorphous at the EM level for ~5–10 h after they became recognizable by light microscopy (n = 2). Nevertheless, by the time the cells reached first mitosis, the second cell cycle centrin aggregates corresponded to morphologically complete centrioles. In all cells investigated (n = 3). Limited sample size did not allow us to identify intermediate stages of the transition from the amorphous centrin aggregates to complete centrioles in cycling cells. During first mitosis, centrioles did not pair to form diplosomes, but rather they organized spindle poles as individual centrioles (Fig. 3), surrounded by minimal amount of PCM.

**De novo-assembled centrioles mature during second cell cycle**

In control HeLa cells, the mother and daughter centrioles exhibit dramatically different mobility during early G1. Whereas the mother centriole remains relatively stationary in the center of the cell, the daughter moves extensively in the cytoplasm making numerous excursions to the periphery of the cell and returning back to the center. These excursions cease at G1/S transition (Piel et al., 2000; Video 3, available at http://www.jcb.org/cgi/content/full/jcb200411126/DC1). Our recordings revealed that centrin aggregates also moved extensively in a random fashion throughout first interphase until the cells entered mitosis (Fig. 2 and Videos 1 and 2). During the first mitosis, approximately half of the of the mitotic cells (11/20) exhibited extra cleavage furrows and produced one mononucleated and one micronucleated daughter cell, which implies the assembly of multipolar spindles in cells with de novo–formed centrioles. Furthermore, we directly observed multipolar mitotic spindle in three cells fixed during first mitosis. Serial section EM reconstruction revealed that centrioles were associated with the spindle poles (Fig. 3).

After completion of first mitosis, which on average was longer in cells born without centrioles (2.9 ± 2.2 h; n = 13) than in their centrosomal sisters (2.0 ± 1.9 h; n = 12), the de novo–formed centrioles resumed movements that were similar to those exhibited by the centrin aggregates during the previous cell cycle. Then, at 2–10 h after mitosis, all centrioles suddenly coalesced into a relatively stationary complex (Fig. 2, E–F; and Videos 1 and 2). In all cases, the coalescence itself was relatively rapid, as all centrioles came together in <2 h. Thus, centriole coalescence corresponds to a pronounced change in behavior: from that normally exhibited by the daughter centrioles to the one typical for the mothers. The coalescence of the centrioles always occurred in the second cell cycle.

It has been previously shown that the motilities of the daughter and mother centrioles during G1 correlate with their...
formed centrioles (arrows). (B and C) Two progeny of a cell born without a centrosome that also contain γ-tubulin, none of them is associated with microtubule foci during late G1 (Fig. 4 C). These observations revealed that, while formation of the morphologically complete centrioles occurs during the first cell cycle, they become competent microtubule-organizing centers only during G1 of the second cell cycle.

The difference between mature (mother) and immature (daughter) centrioles is not limited to their ability to organize microtubules. An important step in the maturation process is to gain the ability to give birth to a new daughter centriole (for conceptual review see Mazia, 1987). Thus, if the de novo–assembled centrioles become mature during the second cell cycle, then they should begin to replicate in a normal fashion. We tested this prediction by following the progeny of cells born without centrioles with continuous time lapse microscopy over three consecutive cell cycles. We were able to obtain full three–cell cycle-long pedigrees for two cells born without centrioles. Analyses of these pedigrees revealed several important features of the centriole cycle. First, we found that centrioles formed de novo replicate in the second cell cycle (Fig. 5, cell a, which fortuitously inherited two de novo–formed centrioles, and its progeny aa and ab). Second, we found that the de novo pathway becomes active whenever the resident centrioles disappear from the cell. This was evident from those cases in which only loose concentration at the perinuclear region. Importantly, this difference in microtubule organization was observed even in those cells where the duration of first cell cycle was for some reason prolonged, something that occurs with equal frequency in cells born with and without centrosomes.

In contrast, after coalescence in the second cell cycle, all centrioles were found to be associated with prominent PCM and reside at the focus of microtubule array (Fig. 4, B and C). Although microtubules in HeLa cells are not prominently radial, the foci associated with the de novo–formed centrioles were very similar to those in the surrounding control cells. Even in those cells that by chance inherited only one centriole, this centriole was able to organize a microtubule focus (Fig. 4 B). The appearance of the centrosome in those cells, that by chance inherited two centrioles, was very similar to that in control cells during late G1 (Fig. 4 C). These observations revealed that, while formation of the morphologically complete centrioles occurs during the first cell cycle, they become competent microtubule-organizing centers only during G1 of the second cell cycle.

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which all the de novo–formed centrioles were distributed to only one of the progeny (Fig. 5, cell a; and Figs. S1 and S2). As a result, the sister cell was born without a centrosome (Fig. 5, cell b), not because of laser ablation, but because of centriole misdistribution. This cell exhibited de novo assembly of eight centrioles that were later distributed in a four-and-four fashion between the two progeny in the next mitosis (Fig. 5, cell b and its progeny ba and bb). It is noteworthy that this mitosis was multipolar; however, because of retraction of one of the cytokinesis furrows, it resulted in the formation of two cells—one mononucleated and one binucleated (not depicted). Thus, de novo–assembled centrioles become completely mature in the next cell cycle after their assembly.

**De novo centriole assembly occurs during the S phase**

Thus far, our data revealed that formation of a mature centriole takes place over two consecutive cell cycles in normally cycling cells. However, it remained unclear whether this was simply a time-dependent process or if progression through the cell cycle is required for centriole formation/maturation. To address this question, we conducted centriole ablations in cells arrested in G₁, with lovastatin or S with hydroxyurea.

We found that formation of centrin aggregates did not occur after ablating resident centrioles in cells arrested during G₁ (Fig. 6 A; n = 5). In contrast, cells arrested in S (Fig. 6 B; n = 5) consistently formed numerous centrin aggregates after the resident centrosome was laser ablated. These dots gradually increased in intensity until they were indistinguishable from normal centrioles. The kinetics of this intensity increase was similar to those observed in the cycling cells during the first cell cycle. EM analysis revealed that centrin aggregates developed into morphologically recognizable centrioles in S-arrested cells (n = 2). In one cell, we found that some centrin aggregates corresponded to structures that appeared to be intermediate stages of centriole formation. EM tomography reconstructions of three of the centrin aggregates in this cell revealed that one of the aggregates corresponded to an electron dense amorphous cloud, with just two microtubule blades present within the cloud. The other two centrin dots corresponded to more completed, although still abnormal, centrioles. These structures contained four microtubule blades in one case and six to seven
in the other; however, the triplet blades were not properly organized into closed cylinders (Fig. 7 and Video 4, available at http://www.jcb.org/cgi/content/full/jcb200411126/DC1).

Fluorescence time lapse microscopy of centrin aggregates/centrioles formed in S-arrested cells revealed that they move continuously in the cytoplasm in the same fashion as centrin aggregates/immature centrioles do in cycling cells during the first interphase (Fig. 6 B and Video 5, available at http://www.jcb.org/cgi/content/full/jcb200411126/DC1). This motion continued for as long as we were able to follow S-arrested cells (~50 h), and the aggregates never coalesced into a common complex. In light of our EM data, these observations indicate that the complete process of centriole biogenesis, but not maturation, can proceed to completion during the S phase.

**De novo centriole formation is inhibited by the presence of a single resident centriole**

Our observations indicated that de novo assembly of centrioles in HeLa cells occurs whenever resident centrioles disappear from the cell. The means in which the centrioles vanish from the cell do not appear to be important, for we observed de novo centriole assembly after ablation of resident centrioles as well as in cells that lost their centrioles via misdistribution during mitosis (Fig. 5). The fact that de novo centriole assembly pathway activates whenever resident centrioles are missing implies that cells possess a mechanism that somehow senses the presence of centrioles. In this respect, it is important to determine whether this mechanism monitors the presence of a mature (mother) centriole or whether it is satisfied by any centriole present in the cell. To address this issue, we laser ablated just one mother centriole within the diplosome at a spindle pole during mitosis. As mother centrioles contain greater amounts of centrin than the daughters, the relative intensity of the GFP signal allowed us to distinguish the mother centriole from the daughter in live cells (Fig. 8; Piel et al., 2000, 2001). As a result, after the end of mitosis, one daughter cell was born with normal centrosome, but the other one inherited only one (daughter) centriole. This centriole replicated once during the first cell cycle, and the resulting diplosome was distributed to one of the progeny during second mitosis. As a result, one of the progeny now inherited a normal centrosome (one daughter and one mother centriole), whereas the other one was born without a centrosome. This cell eventually formed five centrioles de novo. Progeny of the control cell (sister of the one born without centriole) exhibited expected centriole replication pattern.

**De novo centriole formation is not a consequence of laser fragmentation of the resident centrosome**

One formally possible complication of the laser ablation approach to centrosome inactivation is that the laser pulses may cause fragmentation of the centrioles and/or the PCM rather than their complete destruction. If so, the de novo assembly of centrosomes might simply reflect the seeding of centrosomes from tiny preexisting fragments of the original centrosome. To unequivocally test this possibility, we used glass needle microsurgery to remove centrosomes from HeLa cells and to determine whether centrioles would form de novo. Needle microsurgery is effective in completely removing the centrosome from interphase BSC-1 fibroblasts (Maniotis and Schliwa, 1991; Hinchcliffe et al., 2001), and this method cannot induce centrosome fragmentation.

We used the GFP centrin signal to identify cells with two centrioles (G1) that were located away from the nucleus. Such cells were then cut with a glass needle so that a piece of cytoplasm containing the centrosome was separated from the rest of the cell (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb200411126/DC1; also see Hinchcliffe et al., 2001). We then followed each karyoplast by phase-contrast video microscopy for 22–72 h; at the end of the video records, we collected three-dimensional (3-D) fluorescence images. Nine karyoplasts were followed through the first division, and seven were fixed 9–12 h after its completion. The duration of mitosis was ~2 h (range 1–6), which is in good agreement with the duration of the first mitosis in cells after laser ablation of centrosomes. Most karyoplasts formed more than one furrow during cytokinesis, which once again was reminiscent of the cytokinesis pattern in cells after centrosome laser ablation, and implied that...
The mitotic spindle in karyoplasts was multipolar. However, extra furrows regressed and all nine karyoplasts ultimately divided just two daughter cells. All daughter karyoplasts contained a variable number (1–5 per cell) of bright centrin/GFP aggregates indistinguishable from those observed after laser ablations (Fig. 9). Importantly, the number of centrin foci was different between daughter karyoplasts, indicating that the distribution of these structures during mitosis was random, as observed for centrioles formed de novo after laser ablation.

The progeny of two karyoplasts were followed through the second cell cycle, second mitosis, and fixed at ~12 h into the third cell cycle. Each of these cells contained bright centrin aggregates clustered together in a common complex, as expected for de novo–formed centrioles after the maturation (unpublished data). Control amputations of large portions of cytoplasm, not containing centrioles, did not alter cell cycle progression, the normal pattern of centriole duplication, indicating that the distribution of these structures during mitosis was random, as observed for centrioles formed de novo after laser ablation.

In contrast, HeLa cells originate from cervical adenocarcinoma, can be grown in suspension, and have functionally suppressed p53 and Rb as well as deregulated cyclin A expression (for review see zur Hausen, 1996). Thus, the ability or inability to progress through G1 in the absence of centrioles appears to correlate with whether the cells are normal or transformed, respectively.

In any case, the important principle emerging from this work is that G1 progression in mammalian somatic cells does not absolutely require a centrosome; neither the centrosome nor any of its activities are an integral part of the pathways that take a cell through G1 into S phase. Such a “relief of dependency” (Hartwell and Weinert, 1989) implies the existence of a surveillance mechanism (i.e., a checkpoint) that monitors a centrosome-dependent function in normal cells as they enter G1.

**Centrioles possess an activity that represses the de novo pathway**

We find that de novo centriole assembly does not take place when there is a single resident centriole in the cell; it occurs only upon the disappearance of all resident centrioles regardless of the means used to eliminate them (i.e., after ablating the resident centrosome with the laser, after removing it from the cell with a micro-needle, or upon mis-distribution of centrioles during mitosis yielding an acentriolar cell). Thus, centrioles possess an activity that suppresses the de novo pathway (Marshall et al., 2001).

This activity may contribute to the way the cell exercises precise spatial, temporal, and numerical control over centriole duplication in the normal cell. Perhaps, the proximal end of mother centrioles contains a “docking site” that stabilizes and accelerates conversion from a centrin aggregate (precentriole) into a centriole. In this respect, we often detected accumulation of a number of minuscule centrin aggregates in control cells during interphase. However, within a few hours of their formation, these aggregates disappeared (unpublished data). Thus, the formation of precentrioles might always occur via a de novo mechanism and is not under precise numerical control. However, only those precentrioles that associate with the mature resident centrioles are stabilized and elaborate triplet microtubules to become daughter centrioles. This mechanism
readily explains why centrioles are normally not capable of replication within a single cell cycle (Wong and Stearns, 2003) because the stabilization site on the mature centriole may not become available again until the cell undergoes mitosis.

**Centriole cycle and the cell cycle are coordinated by distinct events in S and G1.**

Our finding of de novo centriole formation in cycling cells allowed us to follow the development of centrioles from birth to maturity in the absence of resident mother centrioles whose activities could potentially influence the maturation of the daughters. We observed three distinct stages of the centriole development: (1) formation of centrin aggregates (precentrioles); (2) assembly of the 9 triplet microtubules to form morphologically complete centrioles; and (3) centriole maturation (Fig. 10). We find that formation of precentrioles is first manifested as aggregation of centrin, a protein present in the centriolar lumen and required for centriole replication (Paoletti et al., 1996; Midden-dorp et al., 1997). Centrin is abundant in cells and only <10% of the total centrin is associated with the centrosome (Paoletti et al., 1996). Since centrin shows no tendency to aggregate when overexpressed, it is likely that the precentriole formation is seeded by other components such as the centrin-binding protein Sfi1p (Kilmartin, 2005).

The transformation of precentrioles into morphologically recognizable centrioles is a time-dependent process that runs to completion even when the cell is arrested in S phase by hydroxyurea. When cells progress through S phase with normal kinetics, morphologically recognizable centrioles are not seen until cells approach or enter mitosis. However, we note that, in the normal cell cycle, morphologically recognizable daughter centrioles are consistently found significantly earlier, during early S period (Vorobjev and Chentsov, 1982; Kuriyama et al., 1986). This temporal difference suggests that the normal association of precentrioles with the proximal ends of mother centrioles accelerates the assembly of the barrel of nine triplet microtubules.

The third, or maturation stage, of the centriole development corresponds to a transition in the structural and functional properties of the centriole. The process involves recruitment of additional proteins, such as ninein (Piel et al., 2000; Abal et al., 2002), elaboration of appendages, and is functionally manifested as a dramatic change in the mobility and microtubule anchoring capability of the centriole. While daughter centrioles continuously move in the cytoplasm and are not associated with microtubules, the mothers remain relatively stationary in the center of a microtubule focus (Piel et al., 2000). We find that the maturation of the newly formed centrioles occurs only during G1 of the next (after centriole formation) cell cycle. It is important to emphasize, that centriole maturation is not simply a gradual time-dependent process, as de novo–assembled centrioles remain functionally immature for as long as the HeLa cells are arrested in S phase of the cycle in which these centrioles are assembled. We also observed no centrosome maturation in those cells that were naturally delayed in their progression through the cell cycle. Only after completion of mitosis do the centrioles coalesce into an immobile complex and organize a radial microtubule array. Thus, centriole assembly and maturation are coordinated with the cell cycle by distinct events during S and G1 respectively.

**The de novo pathway and cancer.**

Cancerous cells often possess supernumerary centrosomes, a phenomenon known as “centrosome amplification.” The presence of extra centrosomes greatly increases the probability that such cells will assemble multipolar spindles during mitosis and exhibit genomic instability though unequal chromosome distribution (for reviews see Pihan et al., 1998; Brinkley and Goepfert, 1998). Centrosome amplification has been attributed to cleavage failure and/or centrosome reduplication in a given cell cycle (for reviews see Brinkley and Goepfert, 1998; Pihan et al., 1998; Brinkley, 2001; Nigg, 2002; Tarapore and Fukasawa, 2002). Our observations indicate that the de novo formation of centrioles could also contribute to centrosome amplification. Intriguingly, our data suggest that the consequences of the loss of resident centrosome are dramatically different for normal versus transformed cells because only the latter cells escape G1 arrest in the absence of centrioles. Thus, if...
centrosome misdistribution during mitosis produces an acentriolar daughter cell, then normal cells would remain arrested during G1, however, in transformed cells activation of the de novo pathway will directly result in centrosome amplification through the assembly a multiple centrioles in these cells.

Materials and methods

Cell culture and drug treatments
HeLa-C1 (Piel et al., 2000) cells were provided by M. Bornens (Institut Curie, Paris, France). Cells were grown in antibiotic-free DME supplemented with 10% FBS (HyClone). Cells were maintained in a humidified incubator at 37°C in 5% CO2 atmosphere.

2 mM hydroxyurea, 10 μM aphidicolin, or 5 μM lovastatin were added to cells ~15 h before laser microsurgery. All reagents were purchased from Sigma-Aldrich. For laser microsurgery experiments, cells that were grown on coverslips were mounted in Rose chambers in phenol-free L15 medium supplemented with 10% FBS. During experiments, cells were maintained at 37°C either with custom-built Rose chamber heaters.

Laser microsurgery
Laser ablation of centrosomes was performed as described previously (Khodjakov et al., 2000, 2002; Khodjakov and Rieder, 2001). In brief, collimated 7-ns pulses of 532-nm light from Nd/YAG laser (Surelite II) were steered through the lower epi-port of a microscope (model TE2000e, Nikon), equipped with electronically controlled X-Y stage (Ludl) and UniBlitz shutters (Vincent Associates). The beam was focused 60 × 1.4 NA PlanApo lens that was also used for observations. It takes ~10 laser pulses (1 s) to destroy the centrosome. The system was driven by IP Lab software (Scanalytics), and the images were recorded on the cooled CCD camera (CoolSnap HQ; Photometrics). Images were acquired at 50–200-ms exposures.

Glass needle microsurgery
Glass needle microsurgery and time lapse phase-contrast recording were conducted essentially as described in Hinchcliffe et al. (2001). Karyoplasts were followed until 12 h after the first mitosis or until 12 h after the second mitosis, at which points the cultures were fixed and the state of the centrosome in the karyoplasts characterized by 3-D GFP/immunofluorescence.

Long-term imaging
After laser ablation of the centrosome, the position of the experimental cell was marked with a diamond-tip objective scribe (Carl Zeiss MicroImaging, Inc.). This allowed us to reidentify the cell when the chamber was shuttle between high- and low-power microscopes as well as after fixing the cell for subsequent EM or immunofluorescence analyses.

We used two different strategies to record long-term (up to 85-h) time lapse sequences. In some experiments, phase-contrast time lapse images were recorded at lower magnification using a microscope (model F20; Technai) operated at 200 kV. Images were captured based on a cosine 2-range over ±70° range using a 2k × 2k CCD camera (Gatan) at a pixel size of 3.2 nm. IMOD was used for image alignment and tomographic computations (http://bio3d.colorado.edu/imod).

Online supplemental materials
Fig. S1 shows mitosis in a cell with only one diplosome. Fig. S2 shows removal of centrosome by glass needle microsurgery. Fig. S3 shows selected frames from the time lapse recording of the cell depicted in Fig. 8. Video 1 shows the behavior of de novo-formed centrioles during the first and second cell cycles in HeLa cells. Video 2 provides another example of the de novo-formed centriole behavior during the first and second cell cycles in HeLa cells. Video 3 presents the behavior of resident centrioles during normal cell cycle in HeLa cells. Video 4 presents a walk-through the tomographic reconstruction of de novo–forming centrioles. Video 5 shows the behavior of centrioles formed de novo in a HeLa cell arrested in S with hydroxyurea. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb200411126/DC1.

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