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

Alexey Khodjakov, Conly L. Rieder, Greenfield Sluder, Grisel Cassels, Ody Sibon, and Chuo-Lung Wang

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 and Sluder, 2001). 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 G1, diploid cells contain a 2C amount of DNA and two centrioles, whereas during G2, 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)
implies that in form only in association with preexisting mother centrioles, result, along with the fact that new centrioles normally assembly (in association with the maternal centriole). This presence 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 G1, 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 G1 period of the cell cycle

*Abbreviations used in this paper: 3-D, three-dimensional; HU, hydroxyurea; PCM, pericentriolar material.*
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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 PtK1 (rat kangaroo kidney). We also observed that centrosomes fail to regenerate over a 36-h period when destroyed during G1 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.
and were associated with a large number of small vesicles and Golgi cisternae (Fig. 2). Numerous microtubules emigrated 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) \( \gamma \)-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 \( \gamma \)-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 \( \gamma \)-tubulin/GFP cloud (Fig. 3; Fig. 4 I; 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 \( \gamma \)-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).

**Newly formed \( \gamma \)-tubulin foci also contain other centrosomal proteins and organize multipolar spindles during mitosis**

Immunostaining revealed that newly formed \( \gamma \)-tubulin foci also contained pericentrin and ninein, two well-characterized bona fide centrosomal components. The pericentrin distribution was largely identical to that of \( \gamma \)-tubulin (unpublished data). Ninein was concentrated only in parts of the volume occupied by \( \gamma \)-tubulin. The brightest spots in the ninein pattern were usually seen immediately adjacent to the brightest spots in the \( \gamma \)-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). 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.
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
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 $\gamma$-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 $\gamma$-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 $\gamma$-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...
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.

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-
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 mi-
croscopic 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 cen-
trosome was completely destroyed by the laser microsurgery.
We can also rule out a possibility that a piece of PCM, con-
taining γ-tubulin, survived the operation and was not appar-
rent because it was simply photobleached; centrosome-associ-
ated γ-tubulin is in constant dynamic exchange and a
photobleached centrosome recovers 50% of its original in-
tensity 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 cen-
trioles in HU-arrested cells doubles approximately every 20 h (Bal-
czon 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-tubu-
lin foci formed in various regions of the cytoplasm in the ab-
sence 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 cen-
trosome formation remains to be elucidated, our experi-
ments 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 cen-
trosomes, when they are not delayed in S (Hinchcliffe et al.,
2001); cells normally spend less time in S than the time re-
quired to form a centrosome by the de novo formation. It is
also evident from our previous studies that de novo cen-
trosome formation does not occur in G1 (Hinchcliffe et al.,
2001; Khodjakov and Rieder, 2001). Although we have not
directly tested whether the de novo formation can occur dur-
ing G2, it has been shown that centrioles do not replicate in
cells arrested in G2 for >20 h (Balczon et al., 1995). These
data suggest that de novo assembly of the centrosome re-
quires 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-tubu-
lin–enriched focus, can be detected only 4–5 h after abla-
tion. This delay could indicate a need for a specific gene
expression and/or protein synthesis. Although identifying

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.
putative genes and proteins involved in the process of centriosome 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 Bobinnc 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, Bobinnc 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 Bobinnc et al. (1998) paper and our data, reported here, suggest that the observations of Bobinnc 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 Bobinnc 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 Bobinnc 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 G1 (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 G1.
Time-lapse images were then captured every 15 min for 24 h using a video-rate CCD camera (Model 100; Paulette 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 Mg2+, 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-pericentriolar (gift of Dr. S. Dossey, 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 core facilities.

Our thanks to Mr. R. Cole (Wadsworth Center) for assistance with laser microscopy, were permeabilized with 1% Triton X-100 in PEM buffer (100 mM Pipes, 1 mM EGTA, 5 mM Mg2+, 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-pericentriolar (gift of Dr. S. Dossey, 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.

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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.

References


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