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Centrin-2 Is Required for Centriole Duplication in Mammalian Cells

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Summary

Background: Centrosomes are the favored microtubule-organizing framework of eukaryotic cells. Centrosomes contain a pair of centrioles that normally duplicate once during the cell cycle to give rise to two mitotic spindle poles, each containing one old and one new centriole. However, aside from their role as an anchor point for pericentriolar material and as basal bodies of flagella and cilia, the functional attributes of centrioles remain enigmatic.
Results: Here, using RNA interference, we demonstrate that "knockdown" of centrin-2, a protein of centrioles, results in failure of centriole duplication during the cell cycle in HeLa cells. Following inhibition of centrin-2 synthesis, the preexisting pair of centrioles separate, and functional bipolar spindles form with only one centriole at each spindle pole. Centriole dilution results from the ensuing cell division, and daughter cells are "born" with only a single centriole. Remarkably, these unicentriolar daughter cells may complete a second and even third bipolar mitosis in which spindle microtubules converge onto unusually broad spindle poles and in which cell division results in daughter cells containing either one or no centrioles at all. Cells thus denuded of the mature or both centrioles fail to undergo cytokinesis in subsequent cell cycles, give rise to multinucleate products, and finally die.
Conclusions: These results demonstrate a requirement for centrin in centriole duplication and demonstrate that centrioles play a role in organizing spindle pole morphology and in the completion of cytokinesis.

Introduction

The centrosome is a fascinating organelle that functions as the favored microtubule-organizing framework of eukaryotic cells. The mammalian centrosome normally consists of a pair of microtubule-based centrioles and surrounding pericentriolar material [1]. Centrioles duplicate once during each cell cycle in a process that is initiated at about the time of the G1/S transition and is completed prior to the onset of mitosis, such that, in dividing cells, the two spindle poles that organize the mitotic apparatus each contain a pair of centrioles [2]. However, aside from their role as an anchor point for pericentriolar material [3] and as basal bodies of flagella and cilia [4, 5], the functional attributes of centrioles remain a mystery [6].

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Microsurgical removal or laser ablation of centrosomes results in errors in cytokinesis and in G1 arrest, implicating a role for this organelle in the regulation of cell cycle progression [7, 8]. Centrosome defects (i.e., centrosome amplification and the accumulation of supernumerary centrioles) are characteristic of many solid tumors and may be responsible for the origin of mitotic spindle abnormalities, chromosomal instability, and aneuploidy seen in cancer [9–11]. Furthermore, recent studies showing transient centrosome association of key cell cycle regulators, including the tumor suppressor proteins p53, BRCA-1, and -2, and the cyclin/cdks, have led to speculation that centrosomes provide an important structural context for coordinating cell cycle regulation [12–18]. Taken together, these observations suggest the existence of a centrosome-based checkpoint that functions to monitor coordination between centrosome duplication and DNA replication [19, 20].

The centrins are small calcium binding proteins that are ubiquitous centrosome components [21]. Centrin is one of about 350 "signature" proteins that are unique to eukaryotic cells but have no significant homology to proteins in Archaea and bacteria [22]. Conditional mutations in *CDC31*, the yeast centrin gene, result in failure of the yeast spindle pole body (i.e., the yeast centrosome) to duplicate and result in cell cycle arrest [23, 24]. Genetic studies in the alga *Chlamydomonas* and experimental ablation of centrin synthesis in the cryptogamous water fern *Marsilea* also implicate a key role for centrin in centriole biogenesis [25–27]. Humans and mice have three centrin genes: Cetn-1, which is exclusively expressed in male germ cells, and Cetn-2 and Cetn-3, which are expressed in somatic cells [28–32]. Centrin-2 is a centriole protein, and recombinant GFP-centrin-2 localizes to centrioles throughout the cell cycle, while centrin-3 localizes to the pericentriolar material that surrounds the centrioles [33–36].

Results and Discussion

To address the role of centrin-2 in centriole duplication during the HeLa cell cycle, we exploited the RNA interference technique using a small inhibitory double-stranded RNA homologous to a 21-nucleotide sequence unique to the human centrin-2 message (hCetn-2 siRNA) to reduce centrin-2 expression [37]. Transfection of HeLa cells with hCetn-2 siRNA effectively ablated centrin-2 expression and resulted in a greater than 90% decrease in centrin-2 levels, as determined by Western blot analysis and densitometry on whole-cell lysates (Figure 1A). To assess the possibility of a global effect of the hCetn-2 siRNA treatment on protein expression, we probed parallel blots of the same cell lysates for another centrosome protein, γ-tubulin, and for another calcium binding protein, calmodulin, which shares $\sim$45% sequence identity with centrin (Figure 1A). No reduction in γ-tubulin or calmodulin abundance was detected at 48 or 72 hr following hCetn-2 siRNA treatment

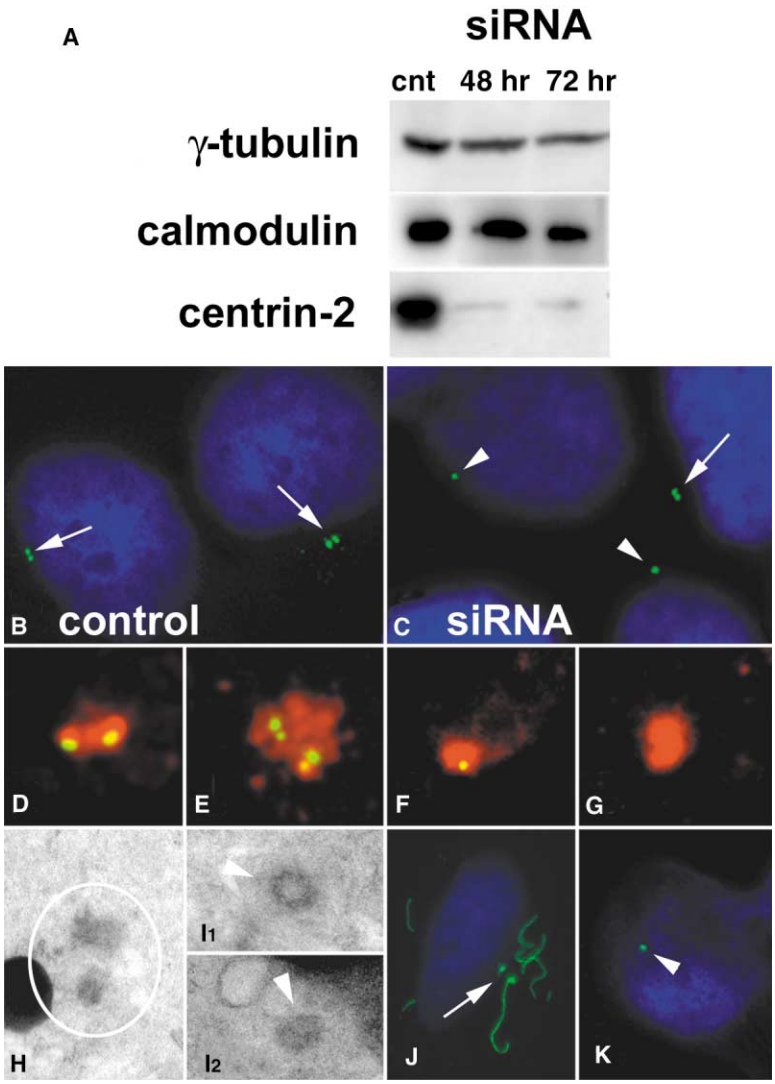

Figure 1. RNA Interference Ablates Centrin-2 Synthesis and Inhibits Centriole Duplication

(A) Western blot analysis of HeLa whole-cell lysates from mock-transfected control (cnt) and hCetn-2 siRNA-transfected (siRNA) 48 and 72 hr samples probed for γ-tubulin (Sigma monoclonal GTU-88), calmodulin (Sigma monoclonal cocktail 2D1 + 6D4 + 1F11), or centrin-2 (monoclonal αCetn2[29]).

(B) Centrin-2 localization showing two αCetn-2-labeled centrioles (arrows) in each of two control cells.

(C) Centrin-2 localization in hCetn-2 siRNA-treated cells (48 hr) showing two αCetn-2-labeled centrioles (arrow) in one cell and a single centriole (arrowheads) in each of the two cells.

(D and E) High-magnification dual indirect immunofluorescence images of centrosomes showing centrioles (green/yellow) and pericentriolar material (red) labeled with monoclonal antibody αCetn2 and rabbit serum 26/14-1, respectively. Control cells show pericentriolar material with (D) two or (E) four centrioles, depending on the cell cycle stage.

(F and G) Examples of centrosomes from hCetn-2 siRNA-treated (48 hr) cells showing pericentriolar material with (F) one centriole and (G) no centrioles.

(H and I) Electron micrographs from serial thick (0.5 μm) sections spanning entire cells showing an example of an (H) orthogonal pair of centrioles (circled) in a control cell and a (I_1, I_2) single centriole (arrowhead) of centrosomes from two different hCetn-2 siRNA-treated cells (48 hr).

(J and K) Anti-tubulin staining of detergent-extracted cells show (J) two centrioles (arrow) in a control cell and a (K) single centriole in a hCetn-2 siRNA-treated (48 hr) cell. Nuclei were stained blue with Hoechst dye ([B] and [C], [J] and [K]).

(Figure 1A). Likewise, no effect of hCetn-2 siRNA treatment was detectable in Coomassie blue-stained gels of the same preparations (not shown). Control transfections with Oligofectamine alone or with siRNA of scrambled sequence were carried out in parallel to experimental treatments and showed no effect on hCetn-2 expression or on the disposition of centrioles (see below). These observations indicate that the siRNA treatment effectively and specifically reduced the abundance of centrin-2 protein.

Dual indirect immunofluorescence labeling employing two different centrin antibodies was used to visualize centrioles and surrounding pericentriolar material in HeLa cells. Centrioles were labeled with the monoclonal antibody αCetn2, which is specific for centrin-2 [30], and pericentriolar material was labeled with the pan-centrin rabbit serum (26/14-1) [35], which recognizes both centrin-2 and centrin-3. As expected, these two antibodies labeled the centrosomes of control HeLa cells and revealed two or four centrin-2-containing centrioles, depending on the stage of the cell cycle, which were located within a more pervasive juxtanuclear accumulation of pericentriolar material (Figures 1B, 1D, and 1E). In contrast, a conspicuous reduction in the number of centrioles was apparent by 48 and 72 hr following hCetn-2 siRNA treatment in which individual HeLa cell centrosomes showed two, one, or no centrioles, while pericentriolar material remained readily detectable (Figures 1C, 1F, and 1G). Quantitative analysis revealed a progressive reduction in centriole number in interphase cells over time following hCetn-2 siRNA treatment (Figure 2). Possible mechanisms leading to centriole loss are discussed below. Additionally, we evaluated centriole number, independent of centrin-2 localization, by using electron microscopy and by indirect immunofluorescence of α-tubulin in cells that had been detergent extracted and cold treated to depolymerize cytoplasmic microtubules. Electron microscopy of serial thick sections spanning entire cells confirmed the presence of orthogonal centriole pairs in control cells (Figure 1H), while several hCetn-2 siRNA-treated cells that were carefully analyzed showed only a single centriole (Figures $1I_1$ and $1I_2$). In experiments in which the centriole number was assessed by tubulin staining, we observed

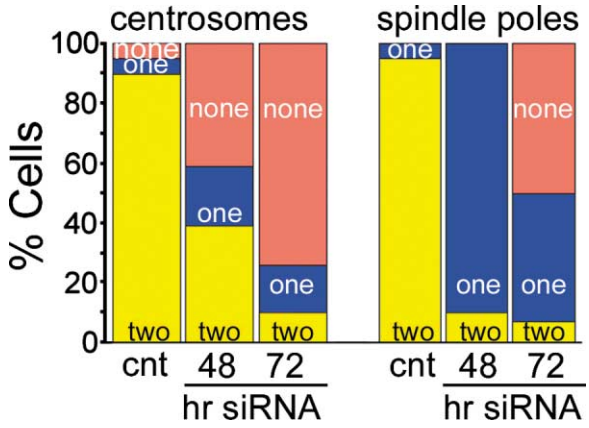

Figure 2. Cells Show a Progressive Loss of Centrioles Following hCetn-2 siRNA Treatment

The percentage of cells showing two, one, or no centrioles are represented by the vertical bars. For centrosomes, 200 cells were analyzed for each condition by indirect immunofluorescence for centrin-2 with αCetn2 monoclonal antibody. For spindle poles, 30 spindles were analyzed for each condition: control (cnt), hCetn-2 siRNA-treated cells (48 hr and 72 hr).

an overall reduction in the centriole number in hCetn-2 siRNA-treated cells compared to the controls (Figures 1J and 1K) — similar to that seen with centrin-2 labeling.

Detailed analysis of mitotic control cells with dual centrin labeling and indirect immunofluorescence showed bipolar spindles with a pair of centrioles embedded in the pericentriolar material at each spindle pole (Figure 3A). Remarkably, hCetn-2 siRNA-treated cells also showed bipolar mitotic spindle formation; however, their spindle poles showed a reduced number of centrioles (Figures 3B–3D). For example, bipolar spindles in hCetn-2 siRNA-treated cells were observed with only one centriole at each pole (Figure 3B), with one centriole at one pole and no centrioles at the other pole (Figure 3C), or with no centrioles at either pole (Figure 3D). Pericentriolar material labeled with the pan-centrin rabbit serum (26/14-1) was present at each spindle pole in hCetn-2 siRNA-treated cells regardless of the presence of centrioles; however, the amount of label was appreciably reduced at spindle poles lacking centrioles altogether (for example, Figures 3C, pole #1, and 3D, poles #1 and #2). Quantitative assessment of the centriole number in interphase centrosomes and mitotic spindle poles revealed a progressive reduction in the centriole number over time in the hCetn-2 siRNA-treated cell population (Figure 2).

Taken together, these data suggest that centrioles failed to duplicate following hCetn-2 siRNA ablation of centrin-2 synthesis. They also suggest that, in hCetn-2 siRNA-treated cells, the preexisting pair of centrioles separated and established the two poles of spindles that completed mitosis and cytokinesis and gave rise to two daughter cells that inherited only a single centriole. Some of these daughter cells continued through a second and even a third cell cycle, also showing failure of centriole duplication, and proceeded to establish bipolar mitotic spindles with either one or no centrioles at all at their spindle poles. While our observations do not allow conclusive interpretation on this issue, it is possible that only the unicentriolar daughter cells that inherited the original mother (older) centriole completed cytokinesis in the second and subsequent cell cycles, since the mother centriole has recently been implicated in signaling the severing of the connecting midbody in late telophase cells [38, 39].

Mitotic spindle abnormalities were an apparent consequence of the failure of duplication and dilution of centrioles following hCetn-2 siRNA treatment. Tubulin labeling of control cells showed spindle microtubules that were associated with condensed chromatin at the metaphase plate and converged into two tightly focused

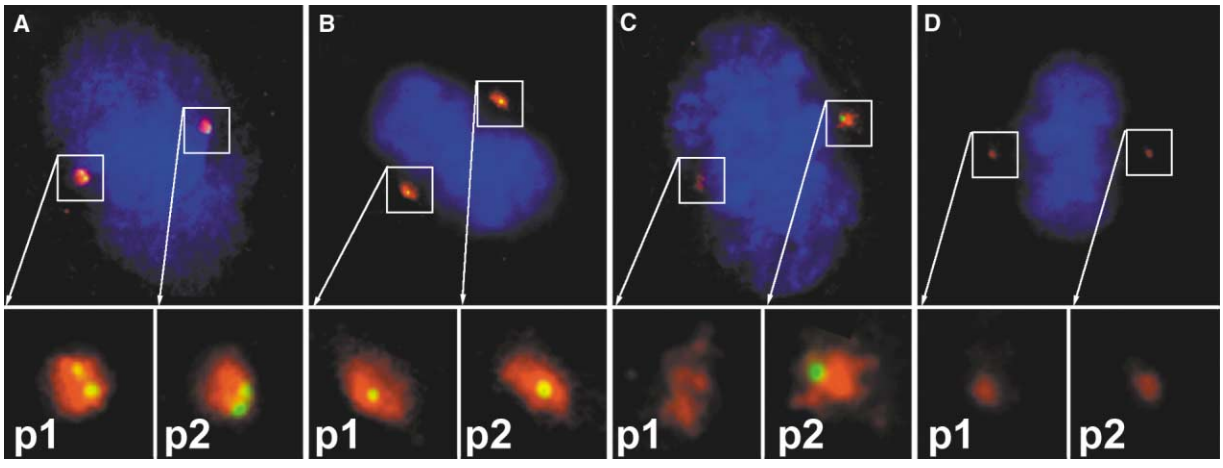

Figure 3. Centriole Dilution Results in Unicentriolar and Acentriolar Mitotic Spindle Poles in hCetn-2 siRNA-Treated Cells

High-magnification dual indirect immunofluorescence showing centrioles (green/yellow) and pericentriolar material (red) labeled with αCetn2 and 26/14-1, respectively.

(A) A control mitotic cell with two spindle poles (p1 and p2), each containing a centriole pair.

(B–D) Bipolar spindles formed in hCetn-2 siRNA-treated cells (48 hr) even though their spindle poles showed an inappropriate number of centrioles. An example of a (B) mitotic cell with one centriole at each pole, a (C) mitotic cell with a single centriole at one pole (p2) and no centrioles at the other pole (p1), and a (D) mitotic cell with no centrioles at either pole are shown. DNA was stained with Hoechst dye (blue).

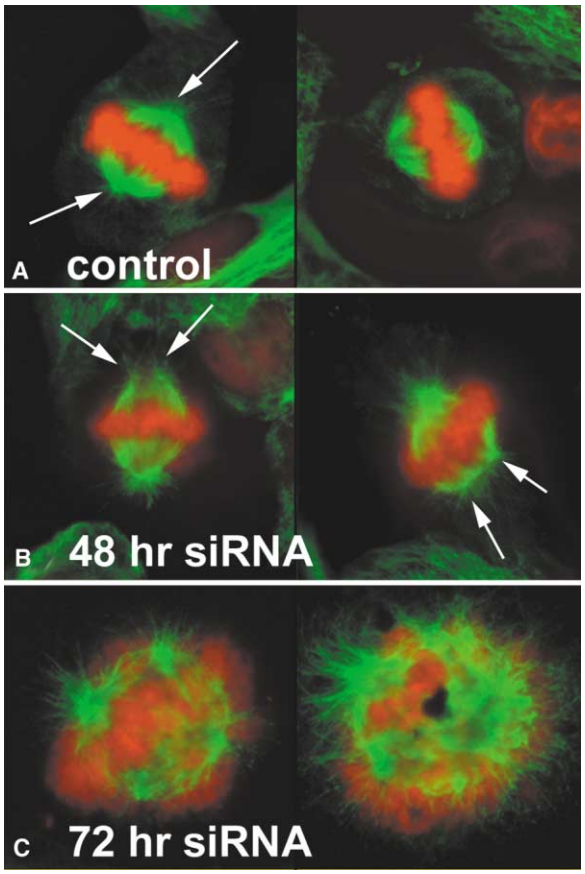

Figure 4. Microtubules Converge onto Unusually Broad Spindle Poles in hCetn-2 siRNA-Treated Cells

(A) Control cells show a tight focus of microtubules converging at each spindle pole (arrows).

(B) While bipolar spindles form in hCent-2 siRNA-treated cells (48 hr), they showed poorly focused convergence of microtubules at broad poles (arrows).

(C) Multipolar spindles predominated in hCent-2 siRNA-treated cells at later times (72 hr). Tubulin is shown in green, and DNA is shown in red.

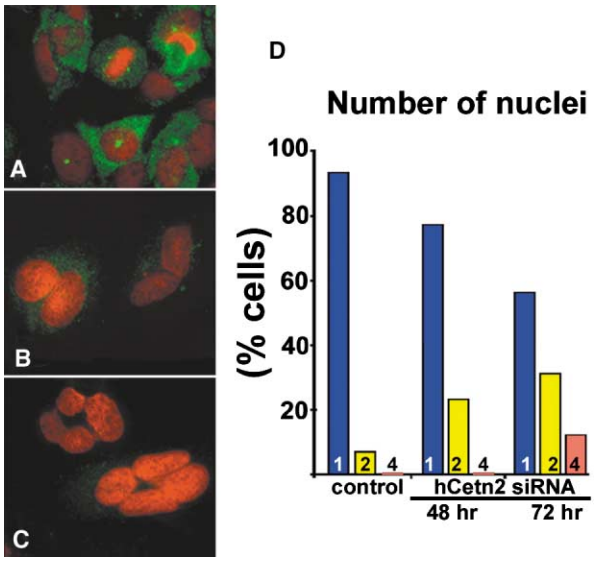

Figure 5. hCetn-2 siRNA-Treated Cells Ultimately Fail to Complete Cytokinesis

(A–C) Control cells showed a (A) single nucleus, while hCent-2 siRNA treatment (48 hr and 72 hr) resulted in an (B and C) increased frequency of multinucleate cells.

(D) Quantitative analysis of the number of nuclei in cells showed a progressive increase of multinucleated cells following hCent-2 siRNA treatment.

spindle poles (Figure 4A). In contrast, while bipolar mitotic spindles formed in hCetn-2 siRNA-treated cells, their spindle poles showed extraordinarily broad and poorly focused convergence of microtubules (Figure 4B). There was, however, a notable overall reduction in the proportion of mitotic cells from ~4%–6% in control populations to less than 1% by 72 hr following hCetn-2 siRNA treatment. In addition, there was an increased frequency of aberrant mitoses, such that, by 72 hr following hCetn-2 siRNA treatment, multipolar mitotic figures were the predominant spindle morphology (Figure 4C). These multipolar mitoses appeared to be a consequence of a failure of the completion of cytokinesis in previous cell cycles, which also resulted in a progressive increase in the proportion of cells with two or four nuclei (Figure 5). Finally, during these later times, there was also a substantial loss of cell adherence and increased cell death, presumably through apoptosis. For example, at 72 hr following hCetn-2 siRNA treatment, only about 40% confluence and a substantial detached cell population was seen compared to control cultures, which were completely confluent and showed few detached cells by this time.

Bipolar mitotic spindles of most animal cells show a tightly focused convergence of microtubules at spindle poles that contain a pair of centrioles. However, bipolar mitotic and meiotic spindles can function in the absence of canonical centriole-containing centrosomes, as is common in higher plants and during mouse oocyte and early zygotic development [40–42]. Moreover, recent experimental studies employing *Drosophila* mutants, in vitro spindle assembly with centrosome-depleted cytoplasmic extracts, or surgical removal of centrosomes in cultured cells indicate that bipolar spindles can form in the absence of functional centrosomes [7, 43–47]. In these systems spindle morphology was typically barrel shaped, anastral, and showed broad spindle poles [40]. These observations demonstrate that, under certain circumstances, centrioles are not essential for spindle assembly and function. However, it is important to recognize that, for cells that normally contain centrosomes, their loss results in increased errors in spindle positioning, cytokinesis, and chromosome loss or missegregation [7, 25, 48, 49]. Similarly, centrosome amplification is a condition seen in many solid tumors that has been implicated as a cause of multipolar mitosis and chromosomal instability, which result in tumor cell heterogeneity during cancer development [9, 10, 50]. With the exceptions indicated above, most eukaryotic cells utilize a centrosome containing a pair of centrioles (or spindle pole bodies) as the favored microtubule-organizing center and exercise exquisite control over the duplication of centrosomes once and only once in each cell cycle.

Conclusions

Here, we demonstrate that centriole duplication in human cells requires synthesis and accumulation of the protein centrin-2. Furthermore, we show that, in the absence of centriole duplication, a preexisting centriole pair can give rise to two functional spindle poles containing only a single centriole; and these spindles can complete at least one round of mitosis and cytokinesis. Daughter cells thus born with only a single centriole may undergo a second and even a third cell cycle (presumably if they are recipients of the original mother centriole). However, progressive failure to complete cytokinesis, accumulation of aberrant multipolar spindles, and ultimately cell death becomes the predominant phenotype for cells blocked in centriole duplication. These observations suggest a function for centrin in centriole duplication and demonstrate that centrioles play a role in organizing spindle pole morphology and in the completion of cytokinesis.

Experimental Procedures

HeLa cells were obtained from ATCC and were grown in DMEM supplemented with 10% fetal calf serum in 5% CO_2 in air at 37°C. Cultures were seeded onto glass coverslips for microscopy or in 6-well, 30-mm plates for cell lysates and Western analysis. For RNA interference, double-stranded RNA homologous to human centrin-2 mRNA coding nucleotides 80–100 (i.e., 5′-AAGAGCAAAAGCAGG AGATCC-3′) was synthesized by Dharmacon Research. Cultures at approximately 30%–40% confluence were transfected in 200 μl antibiotic-free medium with 60 pmole siRNA duplex and Oligofectamine reagent (Invitrogen) for 4 hr, followed by incubation in fresh complete medium. Control transfections used Oligofectamine alone or with dsRNA of scrambled sequence. Standard methods were employed for Western analysis, electron microscopy, and dual label indirect immunofluorescence on methanol-fixed cells [10, 51].

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