Centrobin: a novel daughter centriole–associated protein that is required for centriole duplication

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In mammalian cells, the centrosome consists of a pair of centrioles and amorphous pericentriolar material. The pair of centrioles, which are the core components of the centrosome, duplicate once per cell cycle. Centrosomes play a pivotal role in orchestrating the formation of the bipolar spindle during mitosis. Recent studies have linked centrosomal activity on centrioles or centriole-associated structures to cytokinesis and cell cycle progression through G1 into the S phase. In this study, we have identified centrobin as a centriole-associated protein that asymmetrically localizes to the daughter centriole. The silencing of centrobin expression by small interfering RNA inhibited centriole duplication and resulted in centrosomes with one or no centriole, demonstrating that centrobin is required for centriole duplication. Furthermore, inhibition of centriole duplication by centrobin depletion led to impaired cytokinesis.

Introduction

In mammalian cells, a pair of centrioles and amorphous pericentriolar material constitute the centrosome, which plays a pivotal role in orchestrating formation of the bipolar spindle during mitosis (Kellogg et al., 1994; Zimmerman et al., 1999; Doxsey, 2001). Recent studies have demonstrated that centrosome activity is linked to cytokinesis and the activation of DNA replication (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001; Piel et al., 2001). The latter activity, which is physically associated with core centrosomal structures such as the centrioles or with centriole-associated structures (Hinchcliffe et al., 2001), is probably distinct from the microtubule-organizing activities of the centrosome.

In mammalian cells, the centrioles consist of nine triplets of microtubules, arranged in a cylinder that has a 200-nm diameter and an ~500-nm length, with additional filaments, fibers, and dense material attached to or inside of the microtubular cylinder (Doxsey, 2001; Beisson and Wright, 2003). In addition to α/β-tubulin, only a small number of proteins have been found to localize to the centrioles in mammalian cells. These include centrin, ε-tubulin, centriolin, ninein, and poly(ADP-ribose) polymerase-3 (Salisbury, 1995; Paoletti et al., 1996; Chang and Stearns, 2000; Mogensen et al., 2000; Piel et al., 2000; Ou et al., 2002; Augustin et al., 2003). The centrioles duplicate once per cell cycle in a manner similar to DNA replication. Centriole duplication starts at the onset of the S phase; the two centrioles separate and a new (daughter) centriole forms in association with the mother centriole (Rieder and Borisy, 1982; Vorobjev and Chentsov Yu, 1982). However, exactly how this protein-based structure duplicates accurately remains completely unknown. It has been clearly established that centrosome duplication in mammalian somatic cells requires CDK2 activity (Hinchcliffe et al., 1999; Lacey et al., 1999; Meraldi et al., 1999). Recently, nucleophosmin, Mps1p, and CP110 have been identified as centrosomal substrates of CDK2 and have also been shown to play an important role in the duplication of centrosomes (Okuda et al., 2000; Fisk and Winey, 2001; Chen et al., 2002).

The fidelity of mammalian cell division requires the precise segregation of genetic material into daughter cells, together with a well orchestrated distribution of subcellular organelles. Errors in this process are associated with genetic diseases and underlie aneuploidy, which is a hallmark of cancer progression. Normal centrosome duplication is a key requirement for the correct segregation of chromosomes during cell division, and the centrosome duplication cycle is tightly coupled to the cell division cycle (Doxsey, 2001; for review see Hinchcliffe and Sluder, 2001). Uncoupling of the centrosome duplication cycle from the cell division cycle leads to more than two centrosomes

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Abbreviations used in this paper: cDNA, complementary DNA; HU, hydroxyurea; RNAi, RNA interference; siRNA, small interfering RNA.

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per cell, i.e., centrosome amplification, which is a phenotype frequently observed in both cultured tumor cells and a variety of human tumors (Lingle et al., 1998, 2002; Pihan et al., 1998, 2003; D'Assoro et al., 2002). Thus, the identification and characterization of novel centrosomal proteins that regulate centrosomal duplication is an area of cell biological and medical interest.

We have identified a novel daughter centriole–associated protein that we have designated centrobin (centrosomal BRCA2 interacting protein). RNA interference (RNAi)–mediated depletion of centrobin blocked centriole duplication and led to centrosomes with one or no centriole, demonstrating that centrobin is required for the assembly of the nascent centriole. Inhibition of centriole duplication upon centrobin depletion led to the impairment of cytokinesis. These studies identified a novel component of the daughter centriole that is essential for centriole duplication and function.

Results

Identification, expression, and characterization of centrobin

We identified centrobin in a yeast two-hybrid screening with the conserved COOH-terminal 1,026 residues of BRCA2 as bait. The yeast two-hybrid screening was performed as described previously (Gao et al., 1999, 2000). One set of 11 positive clones encoded the COOH-terminal 539 aa and the 3′-untranslated region of centrobin (Fig. 1, C and E). Northern blotting revealed a single centrobin mRNA transcript of ~3.8 kb expressed in most human tissues and all of the cell lines tested, although the levels varied (Fig. 1, A and B). The basis and significance of the interaction of centrobin with BRCA2 is currently under investigation and is not discussed here.

Two rounds of 5′ rapid amplification of complementary DNA (cDNA) ends PCR, using the human mammary gland Marathon-Ready cDNA as a template, allowed us to clone the 5′ end of centrobin cDNA and to assemble a 3,718-bp cDNA predicting a 903-aa polypeptide (Fig. 1 C). The size of this cDNA, as well as the presence of several in-frame stop codons 5′ to the first in-frame methionine, indicates that this cDNA represents the 3.8-kb transcript observed on the Northern blots (Fig. 1, A and B). One cDNA library-derived clone revealed a 66-bp in-frame insertion after nucleotide 3433, predicting a 925-aa polypeptide (Fig. 1 D). This clone appears to represent a minor transcript, given that only one of the nine sequenced clones contained this 66-bp insertion. In addition, only one EST clone in GenBank contains this insertion (available from Genbank/EMBL/DDBJ under accession no. BM127046). The 3,718-bp transcript was designated as centrobin-α (available from Genbank/EMBL/DDBJ under accession no. AY160226) and the 3,784-bp transcript as centrobin-β (available from Genbank/EMBL/DDBJ under accession no. AY160227). A search of the National Center for Biotechnology Information database indicated that centrobin is a unique protein. Weak homology was observed between the central region of centrobin and various coiled–coil proteins. Centrobin is predicted to have a coiled–coil region at its center and noncoiled regions at the COOH and NH_2 termini, as do some known centrosomal proteins such as pericentrin and ninein (Fig. 1 E).

To examine the expression and function of centrobin, we generated an anti-centrobin antiserum against a His-tagged fusion protein of the COOH-terminal 539 aa of centrobin. To determine whether this antiserum specifically recognizes recombinant and endogenous centrobin, cell lysates from various cell lines and 293T cells transfected with vector, Myc-tagged centrobin, or GFP-tagged centrobin were analyzed by Western

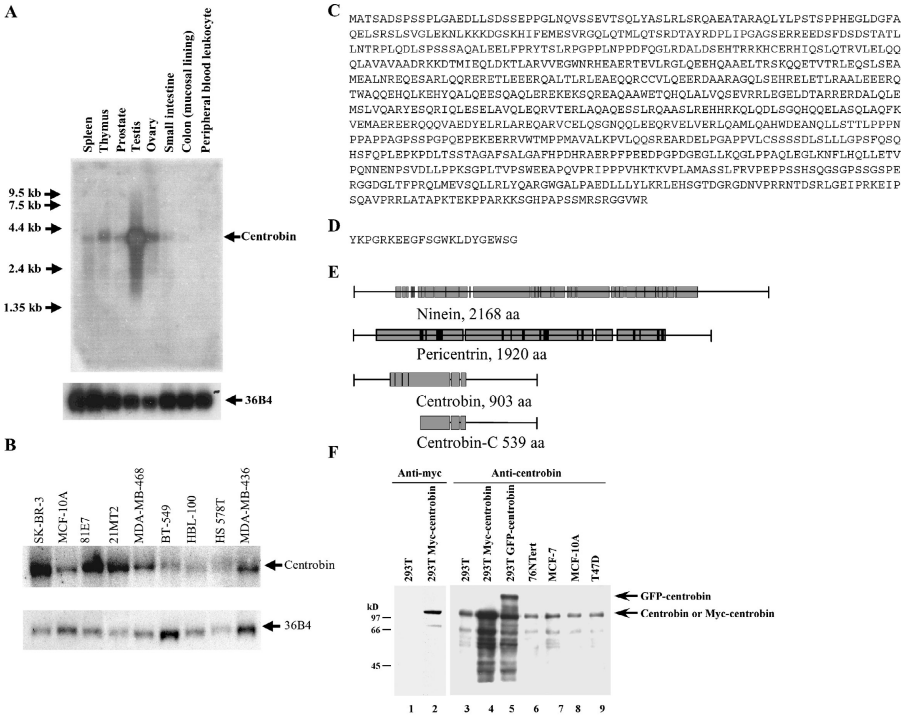


Figure 1. **Centrobin expression, amino acid sequence, and structure.** (A and B) A tissue blot with 2 μg of polyA mRNA per lane (A) and a blot with 20 μg of total mRNA from the indicated breast cell lines (B) were probed with a ^{32}P-labeled centrobin probe followed by autoradiography. Hybridization with the 36B4 probe was used as a loading control. (C) The predicted amino acid sequence of centrobin-α. (D) The extra 22 residues presented only in centrobin-β. (E) Coiled–coil regions of centrobin, ninein, and pericentrin as predicted by DNASTAR. Boxes indicate the coiled–coil region; lines indicate noncoiled regions. Centrobin-C, the COOH-terminal fragment of centrobin that was isolated from the yeast two-hybrid screen. (F) Centrobin protein expression. Cell lysate from the indicated cell lines or 293T cells transfected with pCR3.1 vector, Myc-centrobin, or GFP-centrobin constructs were fractionated by a 6% SDS-PAGE and blotted with affinity-purified anti-centrobin or anti-Myc antibodies.

blotting using affinity-purified anti-centrobin antibody. Consistent with the size of centrobin, the anti-centrobin antibody recognized a 100-kD protein in all cell lines tested (Fig. 1 F, lanes 3–9). As expected, we found that the level of this 100-kD protein is significantly higher in the Myc-centrobin–transfected 293T cells than in the vector-transfected cells (Fig. 1 F, lanes 3 and 4). Anti-Myc antibody specifically detected the 100-kD band only in the Myc-centrobin–transfected cells (Fig. 1 F, lane 2). The specificity of the antiserum was also demonstrated by the presence of a 127-kD protein in the GFP-centrobin–transfected 293T cells (Fig. 1 F, lane 5). Furthermore, preincubating the antibody with purified His-centrobin significantly diminished the specific signal detected by this antiserum (unpublished data). These results demonstrate that the anti-centrobin antiserum specifically detects endogenous centrobin and that centrobin is ubiquitously expressed (Fig. 1 F and not depicted).

Localization of centrobin to the centrosome

The anti-centrobin antibody characterized in Fig. 1 F was used to examine the localization of endogenous centrobin in a normal human mammary epithelial cell line (76N) and several cancer cell lines (T47D, MCF-7, and Capan-1). A typical centrosomal staining pattern was observed in all the cell lines tested, with one or two perinuclear dots in the interphase cells (Fig. 2 A, g, o, and s) and a single focus at the end of each mitotic spindle in mitotic cells (Fig. 2 A, c and k). The centrobin staining pattern was similar to that of γ-tubulin, a protein known to specifically localize to centrosomes (Fig. 2 A, d, h, p, and t). An identical centrosomal staining pattern was observed in MCF10A, HeLa, COS-7, and 293T cells (unpublished data). The centrosomal staining was observed under three different fixation conditions (3.7% formaldehyde, 100% methanol, or 0.5% glutaraldehyde) and also when cells were extracted with 0.5% Triton X-100 in 80 mM Pipes, 1 mM $MgCl_2$, and 5 mM EGTA, pH 6.8, before fixation. Furthermore, the centrosomal localization of centrobin was not affected by treatment with nocodazole (unpublished data). These findings strongly indicated that centrobin is likely to be a bona fide core component of the centrosomes (Oegema et al., 1995). Importantly, GFP-centrobin and Myc-centrobin also localized to the centrosomes in the transfected cells when they were expressed at a very low level (Fig. 2, B and C). It is notable that both GFP-centrobin and Myc-centrobin formed bundle-like structures when expressed at high levels, which is probably an artifact of high-level expression because the endogenous centrobin is expressed at very low levels and is found mainly on centrosomes (unpublished data). A truncated Myc-tagged centrobin (centrobin-C; encoding the COOH-terminal 539 aa) also localized to the centrosomes when expressed at low levels (Fig. 2 D), indicating

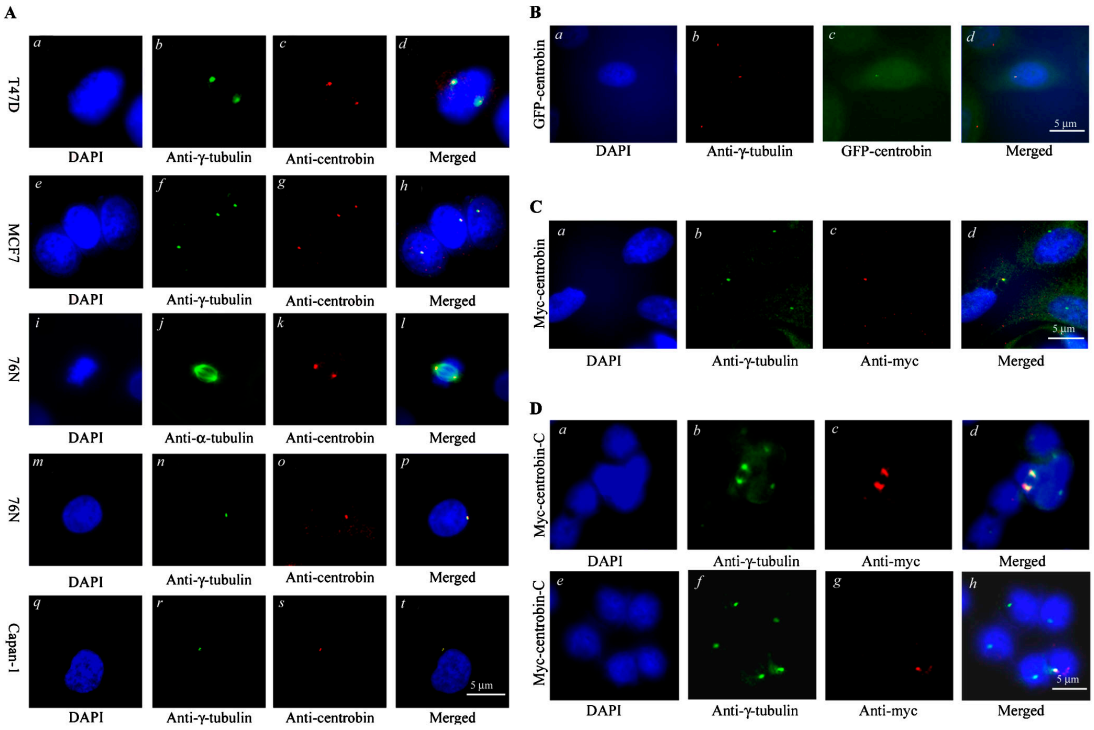


Figure 2. **Localization of centrobin to the centrosomes.** (A) Endogenous centrobin localized to the centrosomes. T47D, MCF-7, 76N, and Capan-1 cells were grown on coverslips, fixed with cold methanol, stained with affinity-purified anti-centrobin (1 μg/ml) and anti–α-tubulin (1:500) or anti–γ-tubulin (1:400), and stained with rhodamine-labeled goat anti–rabbit IgG and FITC-labeled goat anti–mouse IgG. DNA was stained with DAPI. (B) Localization of GFP-tagged centrobin to the centrosomes. 76NTert cells were grown on coverslips, transfected with pEGFP-Myc-centrobin, fixed with cold methanol, stained with anti–γ-tubulin (1:400), and stained again with rhodamine-labeled goat anti–mouse IgG. DNA was stained with DAPI. (C) Localization of Myc-tagged centrobin to the centrosomes. 76NTert cells were transfected with pCR3.1-Myc–centrobin, fixed with cold methanol, and stained with anti-Myc antibody 9E10 (1 μg/ml) and anti–γ-tubulin (1:400), and then with rhodamine-labeled goat anti–mouse IgG and FITC-labeled goat anti–rabbit IgG. DNA was stained with DAPI. (D) Localization of Myc-tagged centrobin-C to the centrosomes. 76NTert cells were grown on coverslips, transfected with pSG5-Myc–centrobin-C, fixed with cold methanol, and stained with anti-Myc and anti–γ-tubulin antibodies (1:400).

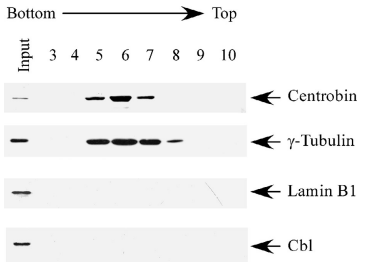


Figure 3. **Copurification of centrobin with the centrosomes through sucrose gradients.** Centrosomes from 293T were purified through a 40–70% sucrose gradient as described (Blomberg-Wirschell and Doxsey, 1998). The resulting fractions were separated on SDS-PAGE gels and blotted with anti-centrobin, anti–γ-tubulin, anti-Lamin B1, and anti-Cbl antibodies.

that the COOH-terminal 539 aa of centrobin is sufficient for centrosomal localization. It is noteworthy that when Myc–centrobin-C was expressed at a high level it decorated the microtubules, which is also probably an artifact of high-level expression (unpublished data).

To corroborate our observation that centrobin localizes to the centrosomes, using immunofluorescence analysis, we biochemically purified the centrosomes from 293T cells using sucrose-gradient sedimentation (Blomberg-Wirschell and Doxsey, 1998) and analyzed their compositions by Western blotting. As shown in Fig. 3, centrobin was found in the fractions that were expected to contain the centrosomes, as confirmed by the presence of γ-tubulin in these fractions (Fig. 3, top two panels). Similar data were obtained using MCF7 cells, which are human breast cancer cells (unpublished data). The centrosomal fractions were shown to be free of nuclear and cytoplasmic contamination by Western blotting using Lamin B1 as a nuclear marker and Cbl as a cytoplasmic marker (Fig. 3, bottom two panels). Collectively, the immunofluorescence and biochemical studies unequivocally demonstrated that centrobin is a centrosomal protein.

Centrobin localizes to daughter centrioles

During our initial immunolocalization analyses we noted that the staining pattern of centrobin differed in cells that appeared to be at different phases of the cell cycle, suggesting the possibility that centrobin may differentially localize in either the mother or daughter centrioles. To further explore this possibility, we performed centrobin localization experiments in synchronized 76NTert cells (an hTert immortalized cell line derived from normal human mammary epithelial 76N cells) in which the two centrioles were typically located farther apart from each other than they are in other cells. Synchronization was achieved by mitotic shake-off. A majority of cells at G0/G1 exhibited a strongly stained centriolar dot, with the other centriole stained weakly or not at all with anti-centrobin antibody. Superimposing centrin and centrobin staining essentially demonstrated complete correspondence of weak centrin staining (daughter centriole) with strong centrobin staining, and vice versa (Fig. 4 A). In the majority of G1/S, S, and G2/M phase cells, there are usually two strongly stained centrobin dots, correlating with the two newly synthesized daughter centrioles, as

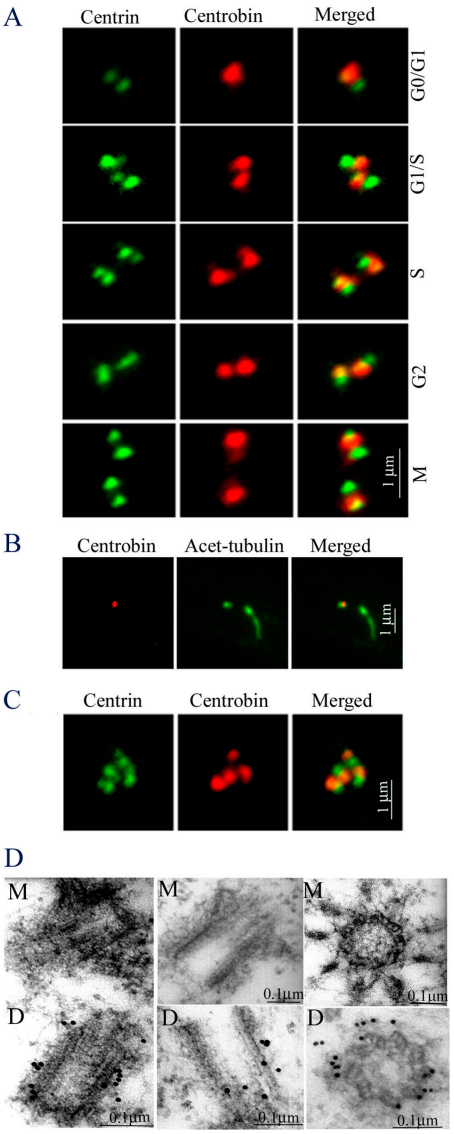


Figure 4. **Centrobin localized to the daughter centriole.** (A) Localization of centrobin during different phases of the cell cycle in 76NTert cells. 76NTert cells were synchronized by mitotic shake-off and replating. Cells were harvested, extracted, fixed with cold methanol, and stained with anti-centrobin (red) and anti–centrin-2 (green) antibodies. (B) Centrobin localization in interphase NIH-3T3 cells. The cells were fixed with cold methanol, and then stained with anti-acetylated α-tubulin (green) and anti-centrobin (red) antibodies. (C) Centrobin localization in U2OS cells treated with HU. U2OS cells were treated with 16 mM HU for 72 h, fixed with cold methanol, and stained with anti-centrobin (red) and anti–centrin-2 (green) antibodies. (D) Immunogold electron microscopic localization of centrobin on the daughter centrioles. 10-nm gold particles were detected on daughter centrioles (D) but not on mother centrioles (M).

indicated by the weaker centrin-2 staining. In some cells, three or four centrobin dots can also be found, including one or two dots with weaker centrobin staining, which are likely to correlate with the original daughter centrioles (mother centrioles, in the current duplication cycle). These findings indicate that centrobin is preferentially incorporated into the newly assembled daughter centriole during centriole assembly at the late G1 or early S phase and that centrobin remains in the daughter centrioles throughout the cell cycle. At the next cycle of centriole

duplication, the amount of centrobin on the original daughter centriole eventually decreases, as shown in the G1/S and S phase cells (Fig. 4 A).

To corroborate these observations, we performed double-immunofluorescence localization of centrobin and acetylated α-tubulin in NIH-3T3 cells. Previous studies have shown that in G0/G1 phase a primary cilium grows from the mother centriole in NIH-3T3 cells and that this cilium can be stained with anti-acetylated α-tubulin antibody (Albrecht-Buehler and Bushnell, 1980; Rieder and Borisy, 1982; Piperno and Fuller, 1985; Lange and Gull, 1995). As shown in Fig. 4 B, anti-centrobin staining clearly superimposed with the centriole without the primary cilium, the daughter centriole. We also analyzed U2OS cells treated with hydroxy urea (HU) for 72 h, a treatment known to induce extensive centrosome amplification (Stucke et al., 2002). As shown in Fig. 4 C, anti-centrobin staining always superimposed with the centrioles with the weaker centrin staining, i.e., the newly synthesized centrioles (Fig. 4 C).

To further characterize the localization of centrobin on the centrioles, we performed immunogold electron microscopy. Nuclear–centrosome complexes were prepared according to the Kuriyama method (Kuriyama and Borisy, 1981) and embedded in LR white resin. Thin sections were incubated with anti-centrobin antibody, followed by incubation with gold-conjugated anti–rabbit secondary antibodies. As in our immunofluorescence studies, centrobin clearly localized to the daughter centrioles, which are the centrioles without the characteristic appendage structure of the mother centriole (Fig. 4 D). We found that centrobin mainly localizes outside of the triplet microtubule blades of the daughter centriole (Fig. 4 D). Some centrobin staining was also found in the lumen and on the triplet microtubule blades of the daughter centriole (Fig. 4 D). Together, these results strongly indicate that centrobin preferentially localizes to the daughter centriole.

Centrobin is required for centriole duplication

We used 21-nt small interfering RNA (siRNA) targeting the coding region of centrobin to knock down centrobin expression. As shown in Fig. 5 A, the endogenous centrobin level was markedly reduced after centrobin siRNA transfection of HeLa cells, but not after the scrambled siRNA and GFP-siRNA were transfected (Fig. 5 A, top). Transfection of HeLa cells with FITC-labeled GFP-siRNA indicated a transfection efficiency of ~90%. Densitometry analysis of the Western blots indicated that 80% of centrobin was reproducibly depleted with centrobin RNAi #1, which we used in all subsequent experiments. The reduction of centrobin levels was also confirmed by immunofluorescence analysis (Fig. 5 B). Immunofluorescence analysis with anti–γ-tubulin antibodies revealed that the γ-tubulin staining pattern was not visibly altered in the HeLa cells with undetectable levels of centrobin (Fig. 5 C). Furthermore, no gross abnormalities in microtubule nucleation and organization was observed in centrobin-depleted cells (Fig. 5 D), suggesting that centrobin likely does not substantially contribute to microtubule organization and nucleation, at least in interphase cells.

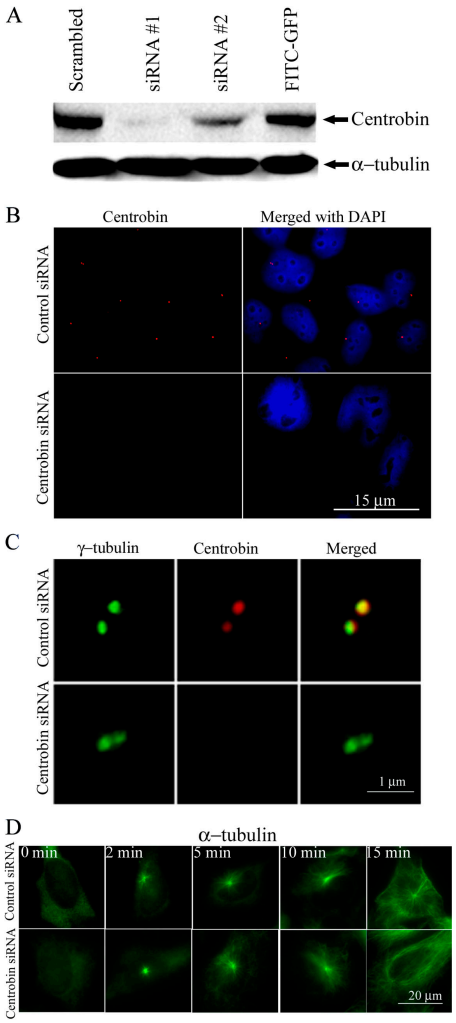


Figure 5. **Centrobin depletion did not affect the localization of γ-tubulin or microtubule organization and nucleation.** (A) Western blot analysis of HeLa cells transfected with centrobin siRNA or control siRNA. HeLa cells were transfected with control (scrambled or FITC-GFP siRNA) or two-centrobin siRNA. After 72 h, cells were harvested and separated through a 6% SDS-PAGE, then blotted with anti-centrobin and anti–α-tubulin antibodies. (B) Immunostaining of centrobin. HeLa cells transfected with scrambled or centrobin siRNA #1 were stained with anti-centrobin and rhodamine-labeled goat anti–rabbit IgG. DNA was stained with DAPI. (C) Centrobin depletion did not affect the localization of γ-tubulin. HeLa cells transfected with scrambled or centrobin siRNA #1 were stained with anti-centrobin and anti–γ-tubulin and with rhodamine-labeled goat anti–rabbit IgG and FITC-labeled goat anti–mouse IgG. (D) Centrobin depletion did not affect microtubule organization and nucleation. HeLa cells were transfected with scrambled siRNA or centrobin siRNA; after 72 h, the cells were treated with 1 μM nocodazole for 1 h and washed three times with PBS to remove the nocodazole. The cells were harvested for fixation using cold methanol and stained for α-tubulin at 0, 2, 5, 10, and 15 min after the removal of nocodazole.

To examine the function of centrobin in centriole duplication, we enumerated the centrioles in control RNAi-treated versus centrobin-depleted HeLa cells using anti–centrin-2 staining. In centrobin-depleted cultures, 21% of interphase cells had four centrioles, 67% had two centrioles, 7% had one centriole, and 2% had no centriole (Fig. 6 A). In contrast, among the control HeLa cells, 47% of the interphase cells had four centrioles, 47% had two centrioles, only 2% of interphase cells had one centriole,

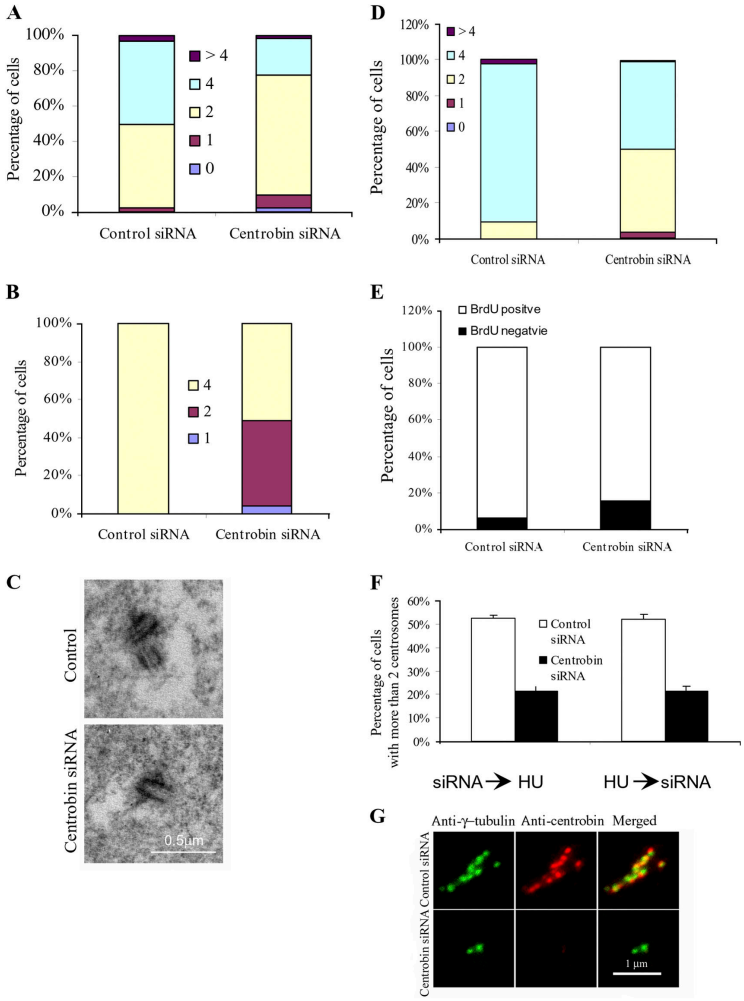


Figure 6. **Centrobin depletion inhibited centrosome duplication.** (A and B) Centrobin depletion inhibited centrosome duplication in interphase and mitotic HeLa cells. HeLa cells were transfected with scrambled siRNA or centrobin siRNA and, 72 h later, were fixed with cold methanol and stained with anti-centrobin and anti–centrin-2 antibodies. The number of centrioles was counted according to the centrin-2 staining in interphase (A) and mitotic cells (B) with an undetectable level of centrobin. Data presented are the percentages of cells with more than four, four, two, one, or zero centrioles (average from three independent experiments). For interphase cells, 300 cells were counted in every experiment. For mitotic cells, 100 cells were counted in every experiment. (C) Electron microscopic examination of centrosomes from control or centrobin-depleted HeLa cells over consecutive thick sections spanning the entire nuclear–centrosome complexes. A representative control cell with two centrioles and a centrobin-depleted cell with one centriole are shown here. (D and E) Centrobin depletion inhibited centriole duplication in HeLa cells arrested by HU. HeLa cells were transfected with scrambled siRNA or centrobin siRNA. 48 h later the cells were treated with16 mM HU for another 24 h, pulse-labeled with BrdU for 30 min, and fixed and stained with anti-BrdU and anti-centrin. Data presented in D are the percentage of cells with four, two, one, or zero centrioles (average from three independent experiments with 300 cells counted in every experiment). Data presented in E are the percentages of cells with positive or negative BrdU staining (average from three independent experiments with 300 cells counted in every experiment). (F) Centrobin depletion inhibited centrosome overamplification in U2OS cells treated with HU. U2OS cells were either treated with HU for 16 h, then transfected with scrambled siRNA or centrobin siRNA, and incubated for an additional 48 h in the presence of 16 mM HU (HU→siRNA) or transfected with scrambled siRNA or centrobin siRNA, and 8 h later treated with 16 mM HU for an additional 62 h (siRNA→HU). All cells were fixed and stained with anti-centrobin and anti–γ-tubulin antibodies. Data presented are the percentages of cells with more than two centrosomes (average and SD from three independent experiments with 300 cells counted in every experiment). (G) Representative U2OS cells transfected with control or centrobin siRNA.

and 0.3% had no centriole (Fig. 6 A). Thus, centrobin depletion induced a marked reduction in the proportion of cells with four centrioles and, correspondingly, an increase in the proportion of cells with fewer than four centrioles. An even more pronounced difference was seen in mitotic cells. Essentially all mitotic cells in the control culture had four centrioles; in contrast, only 51% of the centrobin-depleted mitotic cells had four centrioles, whereas 45% had two and 4% had one centriole (Fig. 6 B). Thus, RNAi-mediated centrobin depletion dramatically inhibited centriole duplication. Nevertheless, the cells were still able to progress through the cell cycle, at least once or twice, generating cells with one centriole or none.

To confirm that the anti–centrin-2 staining dots indeed represent centrioles, we costained the cells with anti–α-tubulin and anti–centrin-2 after the cells were cold treated and detergent extracted to depolymerize the microtubules. We found that anti–centrin-2 staining correlated well with anti–α-tubulin staining (unpublished data). And the centriole number assessed by anti–α-tubulin staining correlated with the centriole number assessed by anti–centrin-2 staining. We also examined the number of centrioles in control and centrobin-depleted cells using electron microscopy. By examining consecutive thick sections spanning entire nucleus–centrosome complexes, we discovered that out of 58 centrobin-depleted cells examined, 1 cell had 5 centrioles, 15 cells had 4 centrioles, 34 cells had 2 centrioles, 5 cells had 1 centriole, and 3 cells had no centriole. A total of 33 control cells were also examined, and out of these 1 cell had 6 centrioles, 19 cells had 4 centrioles, 13 cells had 2 centrioles, and 0 cells had 1 or no centriole. This finding is similar to what was observed using anti–centrin-2 staining. Our observation of cells with one or no centriole in centrobin-depleted cells but not in control cells clearly indicated that centrobin depletion inhibited centriole duplication, confirming our finding using anti–centrin-2 staining dots to represent centrioles.

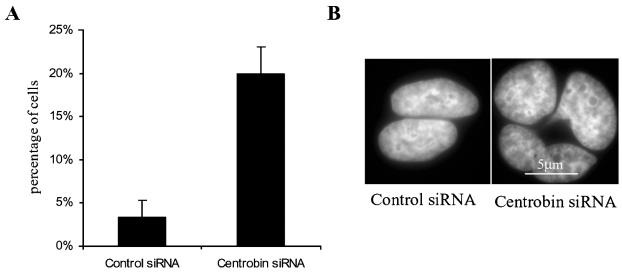
To test whether centrobin depletion directly inhibits centriole duplication, we examined the effect of centrobin depletion on HeLa cells arrested in the S phase. 48 h after siRNA transfection, the cells were exposed to HU for 24 h to induce S phase arrest. The cells were incubated for 30 min with BrdU, harvested, and stained with anti-BrdU and anti-centrobin antibodies. BrdU staining demonstrated that 94% of the control cells and 85% of the centrobin-depleted cells were arrested in the S phase, indicating that centrobin-depleted cells are still able to enter S phase (Fig. 6 D). Because centriole duplication occurs during the late G1 and S phases, all cells arrested in the S phase should contain four centrioles. Indeed, a majority (~87%) of the control RNAi-transfected cells contained four centrioles (Fig. 6 E); only 9% of these cells had fewer than four centrioles.

In contrast, only 48% of the centrobin-depleted cells contained four centrioles, and 50% contained fewer than four centrioles (vs. 9% of control cells; Fig. 6 E). These experiments demonstrated that the majority of centrobin-depleted cells are able to enter the S phase, but are unable to undergo centriole duplication. Therefore, the inhibition of centriole duplication upon centrobin depletion is not a consequence of cell-cycle arrest.

Furthermore, we examined the effect of centrobin depletion in U2OS cells. It has been reported that, upon prolonged S phase arrest by HU, the centrosomes in these cells become overamplified (Stucke et al., 2002). If the effect of centrobin depletion on centrosome duplication was a consequence of preventing cells from entering the S phase, then centrobin RNAi would be expected to be effective only when it was transfected into cells before HU treatment (Meraldi et al., 1999). On the other hand, if centrobin has a direct role in centrosome duplication, then centrobin RNAi would be expected to inhibit centriole duplication even when it is introduced into cells after S phase arrest. As shown in Fig. 6 F, centrosome overamplification was inhibited to a similar extent regardless of whether centrobin RNAi was introduced into U2OS cells before or after S phase arrest. We found that ~52% of the cells transfected with control siRNA had overamplified centrosomes, whereas only 21% of the cells transfected with centrobin siRNA had overamplified centrosomes. Together, these findings clearly indicate that centrobin is required for centriole duplication.

Centrobin depletion leads to impaired cytokinesis

When centrobin was depleted in HeLa cells by the use of siRNA, we observed that the percentage of cells with two or more nuclei increased significantly (from 3% in control siRNA-transfected cells to 20% in the centrobin-depleted cells), indicating a failure of cytokinesis in a proportion of the centrobin-depleted cells (Fig. 7, A and B). To further explore this finding, we directly examined the progression of cell division with time-lapse microscopy. For this purpose, HeLa cells were transfected with control or centrobin siRNA. Phase-contrast time-lapse microscopy was initiated 8 h later and continued for 48 h (Videos 1 and 2, available at http://www.jcb.org/cgi/content/full/jcb.200506185/DC1). In the control cultures, 15 of the 23 observed cells completed mitosis within 2 h, and 22 of the 23 cells completed mitosis within 4 h; no cell failed to complete cytokinesis (Fig. 7, C and D). In contrast, out of the centrobin siRNA-transfected cells that went into mitosis, only 2 of 15 observed cells completed mitosis within 2 h, and only 5 of 15 cells completed mitosis within 4 h; 6 of 15 cells failed to do so within 4 h and 4 of 15 cells exited mitosis without finishing cytokinesis. This finding indicates that centrobin depletion impairs cytokinesis.



C

Centrobin depletion led to a high failure rate in cytokinesis

	Control siRNA	Centrobin siRNA
# of cells underwent mitosis	23	15
Finished mitosis within 2hrs	15	2
Finished mitosis within 2- 4hrs	7	3
Finished mitosis more than 4hrs	1	6
# of cells failed cytokinesis	0	4

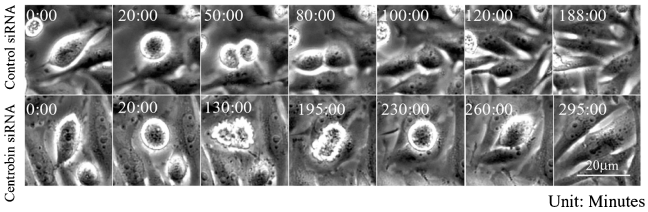


Figure 7. **Centrobin depletion led to impaired cytokinesis.** (A) Centrobin depletion led to cells with multiple nuclei. HeLa cells were transfected with scrambled siRNA or centrobin siRNA and, 72 h later, stained with anti-centrobin and DAPI (blue). The percentage of cells with two or more nuclei was enumerated. Data presented are averages and SDs from three independent experiments with 300 cells counted in every experiment. (B) Representative HeLa cells with two or four nuclei. (C) Centrobin depletion led to impaired cytokinesis. HeLa cells were transfected with scrambled siRNA or centrobin siRNA. 8 h after transfection, time-lapse phase-contrast microscopy was initiated and continued for 48 h. All the cells that underwent cytokinesis were followed to determine the fate and duration of cell division (from cell roundup until two daughter cells separate). The data presented were compiled from two experiments. The durations of cell division for the 23 control cells are 50, 55, 65, 65, 70, 75, 80, 80, 80, 85, 90, 100,100, 105, 110, 140, 160, 165, 170, 180, 200, 220, and 450 min. The durations of cell division for the 11 centrobin-siRNA–transfected cells are 85, 115, 135, 135, 220, 270, 335, 600, 640, 645, and 665 min. Four centrobin-siRNA–transfected cells exited mitosis without finishing cytokinesis at 175, 190, 210, and 260 min. (D) Two representative HeLa cells, one transfected with centrobin siRNA and one with control siRNA that underwent mitosis. The centrobin–siRNA-transfected cell was in mitosis for >260 min and exited without finishing mitosis. The control siRNA transfected cells took <120 min to finish mitosis.

Discussion

We have identified a novel protein, centrobin, which harbors a large coiled–coil domain. We have demonstrated that centrobin is a bona fide core component of the centrosomes. Through detailed analysis of centrobin localization we have established that centrobin asymmetrically localizes to the daughter centrioles. To our knowledge, only poly(ADP-ribose) polymerase-3 has been reported to localize preferentially to the daughter centriole (Augustin et al., 2003). Further studies to identify the binding partners of centrobin will likely reveal more proteins that preferentially localize to the daughter centrioles, which should eventually help to elucidate the mechanism of centriole duplication.

It is of particular importance that the depletion of centrobin in HeLa cells resulted in a high percentage of both interphase and mitotic cells that had two, one, or no centrioles, even under the condition of S phase arrest, which indicates that centrobin depletion inhibits the duplication of centrioles. Further analysis in U2OS cells arrested by HU treatment confirmed

that inhibition of centriole duplication induced by centrobin depletion was not a consequence of cell cycle arrest. These findings have clearly established that centrobin is required for centriole duplication. The exact role of centrobin during centriole duplication remains to be determined. Because CDK2 activity has been shown to be required for centriole duplication (Hinchcliffe et al., 1999; Lacey et al., 1999; Meraldi et al., 1999), it will be of interest to examine if centrobin is a downstream target of CDK2.

We also found that γ-tubulin localization to the centrosomes is not visibly affected in centrobin knockdown cells. Furthermore, centrobin depletion does not appear to affect microtubule organization and nucleation visibly in interphase cells. However, we cannot exclude the possibility that centrobin plays a role in the assembly of mitotic spindles, although we did not observe any conspicuous centrobin staining on the mitotic spindles.

We have also demonstrated that the inhibition of centriole duplication by centrobin depletion leads to the lengthening of mitosis and failure of cytokinesis in a substantial population of cells. The impairment of cytokinesis induced by centrobin depletion is reminiscent of the phenotypes observed in cells when centrosomes were microsurgically removed or laser ablated (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). In those studies, the majority of acentrosomal cells were still able to undergo mitosis but took significantly longer to complete cytokinesis, with a high percentage of cells failing to complete cytokinesis. However, the mechanism of centrobin depletion–induced impaired cytokinesis remains to be determined. Because we did not monitor the status of the centrosome in the recorded cells, we cannot differentiate the effects of centrobin directly on cytokinesis versus impaired cytokinesis because of the formation of acentrosomal spindles.

Thus, centrobin, among a few known centrosome proteins, has a particularly important function in orchestrating centriole duplication. Additional structural and functional analyses are likely to elucidate the mechanistic basis of a fundamental aspect of cell biology, namely, centrosome duplication.

Materials and methods

Cells and media

MCF-7, T47D, U2OS, NIH-3T3, and HeLa cells were grown in α-MEM (Life Technologies) supplemented with 10% FCS (Hyclone). COS-7 and 293T cells were grown in DME (Life Technologies) supplemented with 10% FCS. Breast epithelial cell strain 76N cells, immortal derivative 76NTert cells, and MCF10A cells were grown in DFCI-1 medium (Band and Sager, 1989).

Yeast two-hybrid constructs and screening

The cDNA encoding the COOH-terminal 1,026 residues of BRCA2 was cloned into pGBKT7 (CLONTECH Laboratories, Inc.) to generate the bait plasmid pGBKT7-BRCA2-C3. Yeast two-hybrid screening was performed as previously described (Gao et al., 1999, 2000).

Plasmid constructs and siRNA

The centrobin fragment isolated from the yeast two-hybrid library was cloned into a modified pSG5 vector, pGEX2TK, and into pPROEX Hta to generate Myc-centrobin–C, GST-centrobin–C, and His-centrobin–C. The full-length untagged, Myc-tagged, and GFP-tagged centrobin were constructed by PCR and restriction splicing. The full-length centrobin constructs (untagged, Myc-tagged, and GFP-tagged) used in this study represent centrobin-α. The centrobin-C construct used in this study contains the extra 66-bp of centrobin β. It is referred to only as centrobin elsewhere in this paper.

The siRNAs were synthesized by Dharmacon, Inc. The sequence of centrobin siRNA #1 was AGUGCCAGACUGCAGCAACTT and the sequence of centrobin siRNA #2 was CAACUGGACAAGACCCUGGTT. The sequence of FITC-GFP siRNA was GGCTACGTCCAGGAGCGCACC and the sequence of the scrambled siRNA was CAGTCGCGTTTGCGACTGG. The siRNA transfection was performed with Oligofectamine (Invitrogen) as per the manufacturer's instructions.

Immunofluorescence

Cells grown on coverslips (Fisher Scientific) were fixed in 3.7% PFA/PBS for 10 min at RT, permeabilized in 0.5% Triton X-100/PBS, blocked with 10% goat serum in PBS, and incubated with anti-centrobin, anti-Myc (9E10), anti–α-tubulin, or anti–γ-tubulin (Sigma-Aldrich) in 10% goat serum/PBS. The primary antibodies were detected with FITC or rhodamine-conjugated goat anti–rabbit IgG or goat anti–mouse IgG (The Jackson Laboratory) and the DNA was stained with DAPI. Cells were also fixed in ice-cold methanol for 10 min, in which case the permeabilization step was eliminated. A fixation solution of 0.5% glutaraldehyde was also used. An extraction step before fixation with 0.5% Triton X-100 in 80 mM Pipes, 1 mM $MgCl_2$, and 5 mM EGTA, pH 6.8, was added in some experiments. Gray level images were acquired using a charge-coupled device camera (model ORCA-ER; Hamamatsu) mounted on an epifluorescence microscope (Nikon) and pseudocolored using Adobe Photoshop software.

EM

For immuno-EM, centrosome–nuclear complexes were enriched according to the method described by Kuriyama and Borisy (1981). In brief, 70–80% confluent HeLa cells were scraped off of the culture dishes and pelleted. The cells were resuspended in 10 vol of distilled water for 1 min, an equal volume of lysis buffer (2 mM Hepes and 4% Triton X-100) was added, and the sample was incubated for an additional 5 min. An equal volume of 8% PFA plus 1% glutaraldehyde was added to fix the sample for 15 min at RT. The fixed samples were washed 3 times with Hepes buffer (20 mM Hepes, pH 7.4), dehydrated, and embedded in LR white resin (Ted Pella, Inc.). Thin sections were blocked in blocking buffer (20 mM Hepes, 1% fish gelatin, and 0.4% Triton X-100) at RT for 1 h and incubated with anti-centrobin antibody in blocking buffer (0.5 μg/ml) at 4°C overnight, followed by incubation with goat anti–rabbit IgG conjugated with 10-nm gold particles (Ted Pella, Inc.) for 2 h at RT. The thin sections were further stained with saturated aqueous uranyl acetate and analyzed using a transmission electron microscope (model CM-10; Philips).

To obtain serial thick sections, the purified nuclear–centrosome complexes were fixed in 2.5% glutaraldehyde for 30 min, incubated in 2% osmium tetroxide for 10 min, incubated in 2% uranyl acetate for another 10 min, and embedded in Embed 812. The samples were serially thick-sectioned at 250 or 500 nm and analyzed using a transmission electron microscope (model JEOL-1234; Japan Electron Optics Laboratory Co., Ltd.). A total of 58 centrobin knockdown cells were examined, of which 47 cells were examined using 500-nm sections and 11 cells were examined using 250-nm sections. A total of 33 control cells were examined using 500-nm sections.

Time-lapse imaging

HeLa cells plated on 35-mm dishes with glass coverslip bottoms were transfected with control siRNA or centrobin siRNA. 8 h after transfection, the medium was replaced with CO_2-independent medium (Invitrogen). The cells were imaged every 5 min for 48 h using a 20× phase-contrast lens on an inverted microscope (model TE2000-U; Nikon) equipped with a charge-coupled device camera controlled by Metamorph software (Universal Imaging Corp.). The microscope is enclosed in an incubator box (Life Imaging Services) to maintain a temperature of 37°C.

Online supplemental material

Videos 1 and 2 show that cells transfected with control and centrobin siRNA underwent mitosis and that the silencing of centrobin expression leads to impairment of cytokinesis. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200506185/DC1.

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