SPICE – a previously uncharacterized protein required for centriole duplication and mitotic chromosome congression

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Accepted 25 May 2010
Journal of Cell Science 123, 3039-3046

doi:10.1242/jcs.069963

Summary

Proper assembly and function of a bipolar mitotic spindle is crucial for faithful bidirectional chromosome segregation during cell division. In animal cells, the two poles of the mitotic spindle are organized by centrosomes, microtubule-organizing structures composed of a pair of centrioles surrounded by the so-called pericentriolar material. Proteomic studies have revealed a large number of centrosome proteins, but many remain uncharacterized. Here, we characterize SPICE, a protein that localizes to spindle microtubules in mitosis and to centrioles throughout the cell cycle. RNAi-mediated depletion of SPICE in human cells impairs centriole duplication and causes severe mitotic defects. SPICE depletion compromises spindle architecture, spindle pole integrity and chromosome congression, even in cells in which centriole duplication has occurred. Our data suggest that SPICE is an important dual-function regulator required for centriole duplication and for proper bipolar spindle formation and chromosome congression in mitosis.

Key words: Microtubules, Centrosome, Spindle, Mitosis

Introduction

Bipolarity of the mitotic spindle ensures segregation of the chromosomes into the two daughter cells during cell division. Each spindle pole is formed by a centrosome, a major microtubule-organizing center in animal cells (Luders and Stearns, 2007). Centrosomes are not required for the assembly of a bipolar spindle, but they enhance the efficiency and fidelity of this process (Khodjakov et al., 2000; Basto et al., 2006). Numerical, structural and functional centrosome aberrations can cause spindle abnormalities and result in genomic instability, a phenomenon frequently observed in cancer cells (Nigg, 2006).

The centrosome consists of a pair of centrioles, which dynamically interact with the surrounding proteinaceous matrix, the pericentriolar material (PCM). The PCM promotes microtubule assembly by providing microtubule-nucleation sites and serves as a docking platform for regulatory proteins. In addition, the PCM also plays a role in centriole duplication, and hence centrosome number and propagation (Pelletier et al., 2004; Dammermann et al., 2008; Loncarek et al., 2008).

Duplication of the single centrosome occurs during S phase of the cell cycle, and depends on both PCM and centriolar components (Dammermann et al., 2004). The PCM surrounding the mother centriole recruits regulatory and structural proteins required for the assembly of the new daughter. Polo-like kinase 4 (Plk4) is a key regulator of this process (Bettencourt-Dias et al., 2005; Habedanck et al., 2005). Restricting centrosome duplication to occur only once per cell cycle ensures spindle bipolarity and is tightly regulated (Nigg, 2007). Several regulatory mechanisms have been proposed. The orthogonal engaged configuration of a newly formed mother–daughter pair might prevent further duplication rounds in S phase. Licensing of a new round of duplication involves disengagement of the centriole pair and typically occurs when cells progress through mitosis (Tsou and Stearns, 2006; Tsou et al., 2009). In addition, the availability of cytoplasmic factors required for daughter centriole formation (Strnad et al., 2007; Cunha-Ferreira et al., 2009b; Rogers et al., 2009) and the size of the PCM cloud surrounding the mother centriole (Loncarek et al., 2008) have been suggested to restrict the number of duplication rounds during S phase.

In addition to numerical aberrations, structural centrosome defects can also interfere with spindle bipolarity by causing spindle pole fragmentation (Gergely et al., 2003; Cassimeris and Morabito, 2004; Cho et al., 2006; Oshimori et al., 2006; Haren et al., 2009). Moreover, pole fragmentation and multipolar spindles can be the result of an imbalance of microtubule-dependent forces within the spindle (Wittmann et al., 2000; Garrett et al., 2002; Lawo et al., 2009). In summary, these observations indicate that centrosomes and mitotic spindles are intimately linked at both the structural and functional level.

A large number of proteins associated with specific structures of the microtubule cytoskeleton have been identified (Andersen et al., 2003; Skop et al., 2004; Keller et al., 2005; Sauer et al., 2005), but many still await functional characterization. Assigning functions to these proteins and analyzing their interplay will be crucial for understanding the architecture and regulation of complex microtubule arrays such as the mitotic spindle.

Results and Discussion

In a recent study, fractionation of centrosomes by salt extraction and centrifugation was combined with mass spectrometry to determine how tightly proteins interact with the centrosomal scaffold (Andersen et al., 2003). We were interested in identifying centrosomal protein complexes and reasoned that such proteins would not only co-segregate in the salt extraction assay, but would also show a similar localization pattern in cells. We identified co-segregating proteins based on published data (Andersen et al.,

2003) and screened candidates by expression as enhanced GFP (EGFP) fusions in human cells. This approach identified four proteins that localized to centrioles and to mitotic spindle microtubules: HAUS3, HAUS5 and HAUS6, three subunits of the augmin complex that were recently described by other laboratories (Zhu et al., 2008; Lawo et al., 2009; Uehara et al., 2009); and the previously uncharacterized protein CCDC52, which we describe here. Despite the similar localization pattern, CCDC52 has properties that distinguish it from the augmin complex and we have renamed this protein SPICE (spindle and centriole protein).

SPICE is associated with spindles and centrioles

To confirm that SPICE is a centrosomal protein, we stained human cells with an antibody raised against a recombinant fragment of SPICE and with γ-tubulin antibodies. The anti-SPICE antibody labeled centrosomes throughout the cell cycle and spindle microtubules during mitosis (Fig. 1A). A similar staining pattern was observed with a second antibody that we raised and with a commercial antibody (data not shown). Transiently expressed EGFP-tagged SPICE also localized to centrosomes and spindles (Fig. 1B). Centrosomal SPICE localized to dot-like structures within larger foci formed by the PCM marker γ-tubulin (Fig. 1A). To test whether SPICE might localize to centrioles, we co-stained endogenous SPICE with stably expressed EGFP–centrin or transiently expressed EGFP–SPICE with endogenous centrin (Fig. 1C,D). Foci of centrin, a centriole marker at the distal end of centrioles, were always equal in number and adjacent to the SPICE foci, suggesting that SPICE was present in the proximal part of the centrioles.

Subcellular targeting of SPICE is mediated by two distinct conserved regions

Analysis of the SPICE amino acid sequence did not reveal any functional features based on similarity to other proteins, but identified two potential coiled-coil regions (CC1 and CC2). SPICE-related sequences seem to be present only in vertebrates. Alignment of SPICE sequences from human, mouse, frog and zebrafish showed that both predicted coiled-coil regions are well conserved (Fig. 1E). To learn more about the subcellular targeting of SPICE, we constructed EGFP fusions of three different SPICE fragments comprising N-terminal, central or C-terminal regions (Fig. 1E), and expressed the fusion constructs in human cells. Surprisingly, in interphase cells, the N-terminal fragment (1–450), containing the CC1 region, accumulated in the nucleus, where it formed dot-like aggregates. As nuclear accumulation was not observed for the full-length protein, we have not investigated it in more detail. In mitotic cells, fragment 1–450 localized to centrioles and spindle microtubules (Fig. 1F), similar to full-length EGFP–SPICE (Fig. 1B). The central fragment (350–750), containing CC1 and CC2, strongly localized to centrioles in both interphase and mitosis, but did not show colocalization with spindle microtubules (Fig. 1G). The C-terminal fragment (444–856), containing CC2, did not show any specific localization pattern (Fig. 1H). In summary, the localization studies suggest that distinct regions mediate the targeting of SPICE to spindles and centrioles, and that the central conserved CC1 region is involved in centriole localization.

Depletion of SPICE causes defects in the mitotic spindle and impairs chromosome congression

To study the function of SPICE in human cells, we depleted the protein by RNAi using two different oligonucleotides. Both oligonucleotides efficiently depleted SPICE and produced similar phenotypes. The data shown in this paper were generated with oligo 1 (see Materials and Methods). Western blotting of cell extract indicated that SPICE levels were reduced by ~90% compared with control cells (Fig. 2A). Depletion of SPICE resulted in a 3–4-fold increase in the mitotic index, suggesting impaired mitotic progression in the absence of SPICE (Fig. 2B). We tested whether abnormal spindle formation and/or function was responsible for this defect. Indeed, SPICE depletion caused an increase in the percentage of mitotic cells with multipolar spindles (~25% compared with <5% in control cells; Fig. 2C,D) and, most strikingly, interfered with chromosome congression (Fig. 2C,E). Chromosome congression was impaired even in cells that had assembled bipolar spindles. In ~85% of bipolar-spindle-containing control cells, all chromosomes had congressed to the metaphase plate, whereas only ~15% of cells showed this configuration after SPICE depletion (Fig. 2E). The majority of SPICE-depleted cells displayed congression defects ranging from a few uncongressed chromosomes to a complete lack of congression (Fig. 2C,E). Time-lapse imaging of SPICE-depleted cells expressing Cherry–H2B to label chromatin confirmed that these cells were unable to align their chromosomes (supplementary material Movies 1 and 2). Control cells typically completed mitosis within one hour, whereas SPICE-depleted cells with chromosome alignment defects spent an average of ~5 hours in a prometaphase-like state without progressing to anaphase (n=9 cells). After several hours of mitotic arrest, these cells displayed membrane blebbing and DNA fragmentation, suggesting that they underwent apoptosis.

SPICE is required for centriole duplication

To investigate whether the multipolar spindle phenotype in SPICE-depleted cells was caused by an increase in centrosome number, we stained cells with centrin-specific antibodies as a marker for centrioles, and counted centrin foci in mitotic prometaphase and metaphase cells. About 85% of control cells contained the expected four centrin foci (two pairs of centrioles, one pair at each pole; Fig. 3A,B). Interestingly, SPICE-depleted cells showed a reduction in centriole number: only ~40% of SPICE-depleted cells contained two pairs of centrioles. The majority of cells contained three (2+1), two (1+1) or only a single centriole (Fig. 3A,B), suggesting defective centriole duplication in the previous S phase. This result was confirmed by a centriole overduplication assay. During prolonged S-phase arrest, some transformed cells, such as U2OS cells, undergo several rounds of centriole reduplication (Balczon et al., 1995). Sixty hours after hydroxyurea-induced S-phase arrest, almost 70% of control cells contained more than four centrioles, whereas only ~15% of SPICE-depleted cells had overduplicated their centrioles and most of them had four or fewer centrioles (Fig. 3C).

Consistent with the finding that SPICE depletion does not generate cells with extra centrosomes, co-staining of SPICE-depleted cells with centrin- and γ-tubulin-specific antibodies revealed that the additional spindle poles were formed by acentriolar ectopic foci (Fig. 3A). These ectopic foci were also labeled by antibodies against other PCM components, including GCP-WD, Cep215 and pericentrin (Fig. 3D). Together, these results indicate that the observed spindle multipolarity in SPICE-depleted cells is not the result of increased centrosome number; instead, SPICE depletion interferes with centriole duplication and the formation of multiple spindle poles in mitosis involves acentriolar ectopic PCM foci.

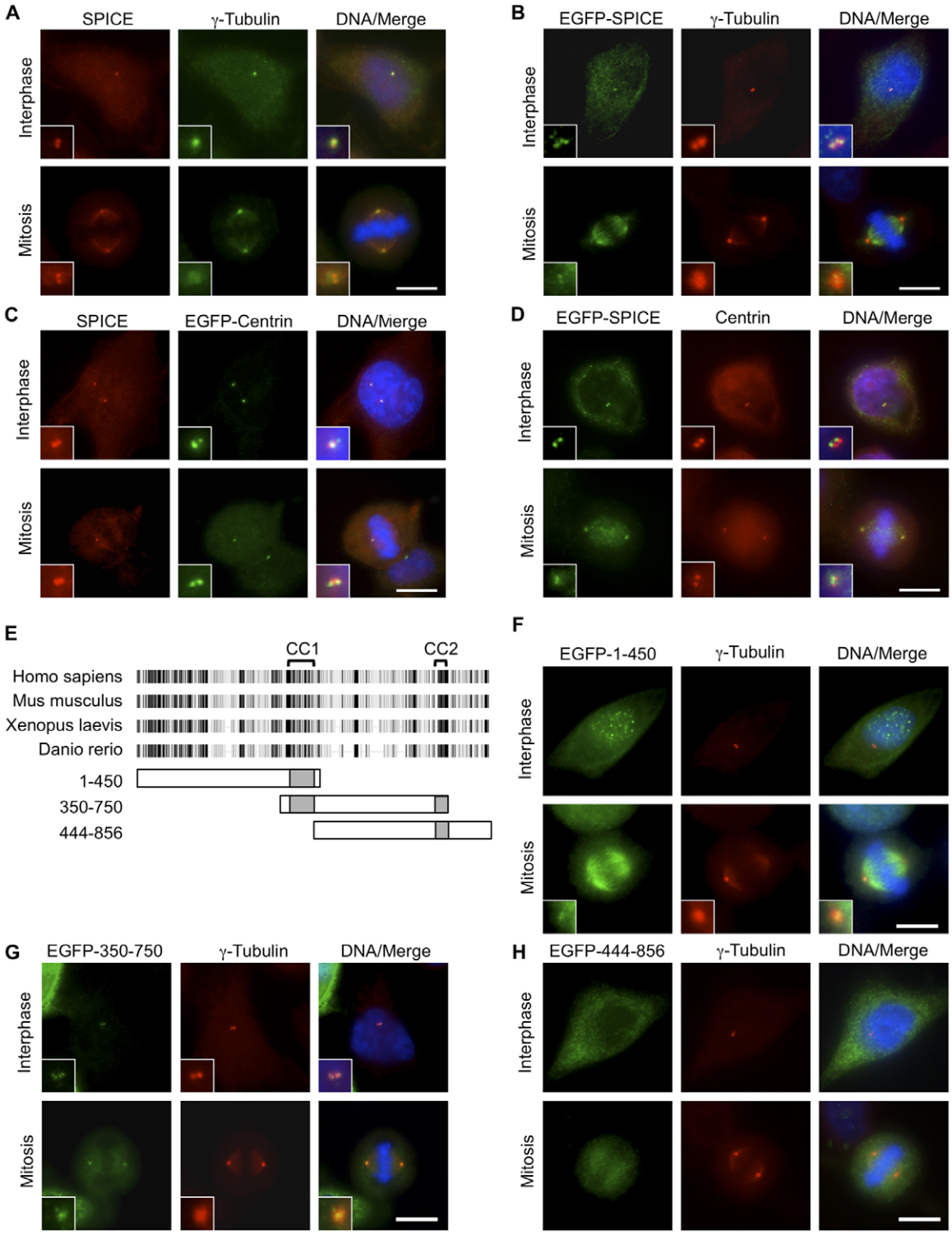

Fig. 1. Two different SPICE regions mediate the localization of SPICE to centrioles and spindle microtubules. (**A**) HeLa cells were co-stained with anti-SPICE and anti-γ-tubulin antibodies. DNA was stained with DAPI. Insets are magnifications of centrosome areas. Scale bar: 10 µm. (**B**) HeLa cells transiently expressing EGFP-tagged full-length SPICE were fixed 48 hours after transfection and stained with antibodies against the EGFP tag and γ-tubulin. DAPI was used to stain DNA. Scale bar: 10 µm. (**C**) U2OS cells stably expressing centrin–EGFP were co-stained with antibodies against GFP and SPICE. DNA was stained with DAPI. Insets show magnifications of the centrosome areas. Scale bar: 10 µm. (**D**) HeLa cells were transfected as in B and co-stained with antibodies against GFP and centrin. DNA was visualized by DAPI staining. Insets are magnified centrosome areas. Scale bar: 10 µm. (**E**) Graphic representation of an alignment of SPICE amino acid sequences from different species. The most highly conserved regions are shaded in black, others in gray. A schematic representation of three different SPICE fragments is shown under the alignment. Numbers represent amino acids. The positions of the two conserved coiled-coil regions (labeled CC1 and CC2) are indicated. (**F–H**) EGFP-tagged SPICE fragments were transiently expressed in HeLa cells and co-stained with anti-GFP and anti-γ-tubulin antibodies. DNA was stained with DAPI. Scale bars: 10 µM.

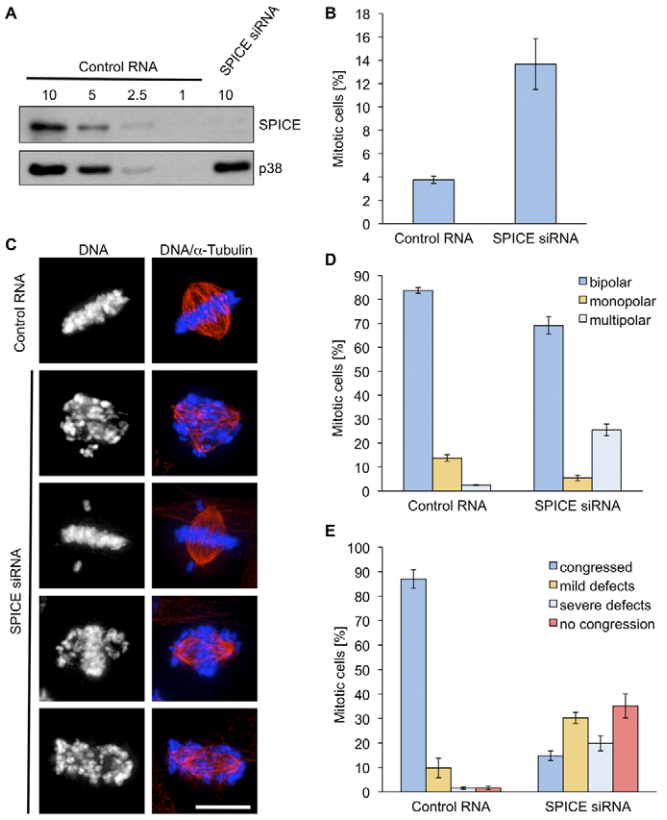

Fig. 2. Depletion of SPICE causes defects in the mitotic spindle and impairs chromosome congression. (**A**) HeLa cells were transfected with control RNA and *SPICE* siRNA. 48 hours later, cell extracts were analyzed by western blotting, and probed for SPICE and the kinase p38 as a loading control. The relative amounts of extract loaded are indicated by numbers above each lane. (**B**) The percentage of mitotic cells in control and SPICE-depleted cells was determined 48 hours after transfection (mean of three experiments; >500 cells counted for each sample; error bars indicate s.e.m., 95% CI). (**C**) HeLa cells were analyzed 48 hours after transfection of control RNA and *SPICE* siRNA. Microtubules were stained with anti-α-tubulin antibodies and DNA with DAPI. Maximum projections of deconvolved z-stacks are shown. Scale bar: 10 µm. (**D**) Distinct spindle morphologies – bipolar, multipolar and monopolar – were scored in cells transfected as in C (mean of two experiments; >100 mitotic cells counted for each sample; error bars indicate s.e.m., 95% CI). (**E**) Cells transfected as in C were scored for mitotic chromosome configurations. Mild and severe defects signify <50% and ≥50% of uncongressed chromosomes, respectively (mean of three experiments; >100 mitotic cells counted for each sample; error bars indicate s.e.m., 95% CI).

In the presence of ectopic foci, centrosomes in SPICE-depleted cells were not always part of the dominant spindle structure (Fig. 3A, Fig. 4B). Quantification of pericentriolar γ-tubulin at centrosomes containing two centrioles indicated a reduction by ~40% (s.e.m. 11.9%, 95% CI, n=15) compared with control cells, and by ~70% (s.e.m. 13.3%, 95% CI, n=20) if only a single centriole was present. One interpretation of this result would be that SPICE is required for the recruitment of PCM to centrioles. Alternatively, SPICE depletion might lead to microtubule-dependent pole fragmentation and ectopic PCM accumulation by generating an imbalance of forces within the spindle. To address this question, we tested the effect of microtubule depolymerization. First, we determined the number of γ-tubulin foci in mitotic control and SPICE-depleted cells. Almost all control cells had the expected number of two γ-tubulin foci. After SPICE depletion, >50% of cells had more than two γ-tubulin foci (Fig. 3E). Treatment with nocodazole largely abolished the additional γ-tubulin foci in SPICE-depleted cells, indicating that generation of ectopic foci is a microtubule-dependent process (Fig. 3E). These results are consistent with the interpretation that SPICE depletion induces an imbalance of forces within the mitotic spindle, which causes microtubule-dependent disruption of spindle poles.

SPICE depletion impairs spindle architecture independent of centriole number

Human cells depleted of Plk4, a key regulator of centriole duplication, assemble functional bipolar spindles and progress through mitosis, despite the presence of spindle poles containing only a single centriole (Habedanck et al., 2005). Severe mitotic defects occur only after at least two consecutive cell cycles and rounds of failed centriole duplication, which generate cells containing only a single centriole and hence a single centrosome. In such cells, monopolar spindles are the predominant phenotype (Habedanck et al., 2005; Bettencourt-Dias et al., 2005). Similar results have been described for human SAS-6, another key component of the centriole duplication pathway (Leidel et al., 2005). Moreover, cells progress through mitosis and undergo cytokinesis even in the complete absence of centrioles (Khodjakov et al., 2000; Basto et al., 2006). We concluded that impaired centriole duplication might not qualify as the sole cause of the severe mitotic defects seen in SPICE-depleted cells and that SPICE might have additional important functions during mitosis. In support of this view, we also observed mitotic defects in the fraction of SPICE-depleted cells that had a normal centriole number (judged by the presence of two centrin foci at each pole): bipolar spindles were abnormally long (11.1 µm compared with 8.8 µm in control cells; Fig. 4A); and occasionally ectopic PCM foci and chromosome congression defects were present (Fig. 4B).

Chromosome congression and alignment in metaphase involves interaction of kinetochores with K-fibers, microtubule bundles connecting the kinetochores to the spindle poles. Compromised kinetochore function impairs chromosome congression by interfering with microtubule capture and proper K-fiber formation (DeLuca et al., 2002; Martin-Lluesma et al., 2002). We tested whether SPICE-depleted cells contain well-defined kinetochores. CREST staining, which labels components of the inner kinetochore (Brenner et al., 1981), revealed the presence of kinetochores in both control and SPICE-depleted cells (supplementary material Fig. S1). CENP-E, an outer kinetochore component required for chromosome congression (Wood et al., 1997; Yao et al., 1997), was also present at kinetochores in SPICE-depleted cells. Interestingly, CENP-E was mostly detected at kinetochores of unaligned chromosomes and strongly reduced at aligned chromosomes (supplementary material Fig. S1). The presence of both CREST and CENP-E staining at unaligned kinetochores suggests that the congression defect is not simply caused by the lack of kinetochore structures. K-fiber formation also involves spindle-associated factors such as the augmin complex and the protein HURP, which stabilizes K-fibers and localizes specifically to the plus ends of the K-fiber microtubules in close proximity to the chromosomes (Koffa et al., 2006; Sillje et al., 2006; Wong and Fang, 2006). We used HURP staining as a marker to test whether the chromosome congression defects in SPICE-depleted cells might involve aberrant K-fibers. We quantified the amount of spindle-associated HURP along the axis of bipolar spindles. In control

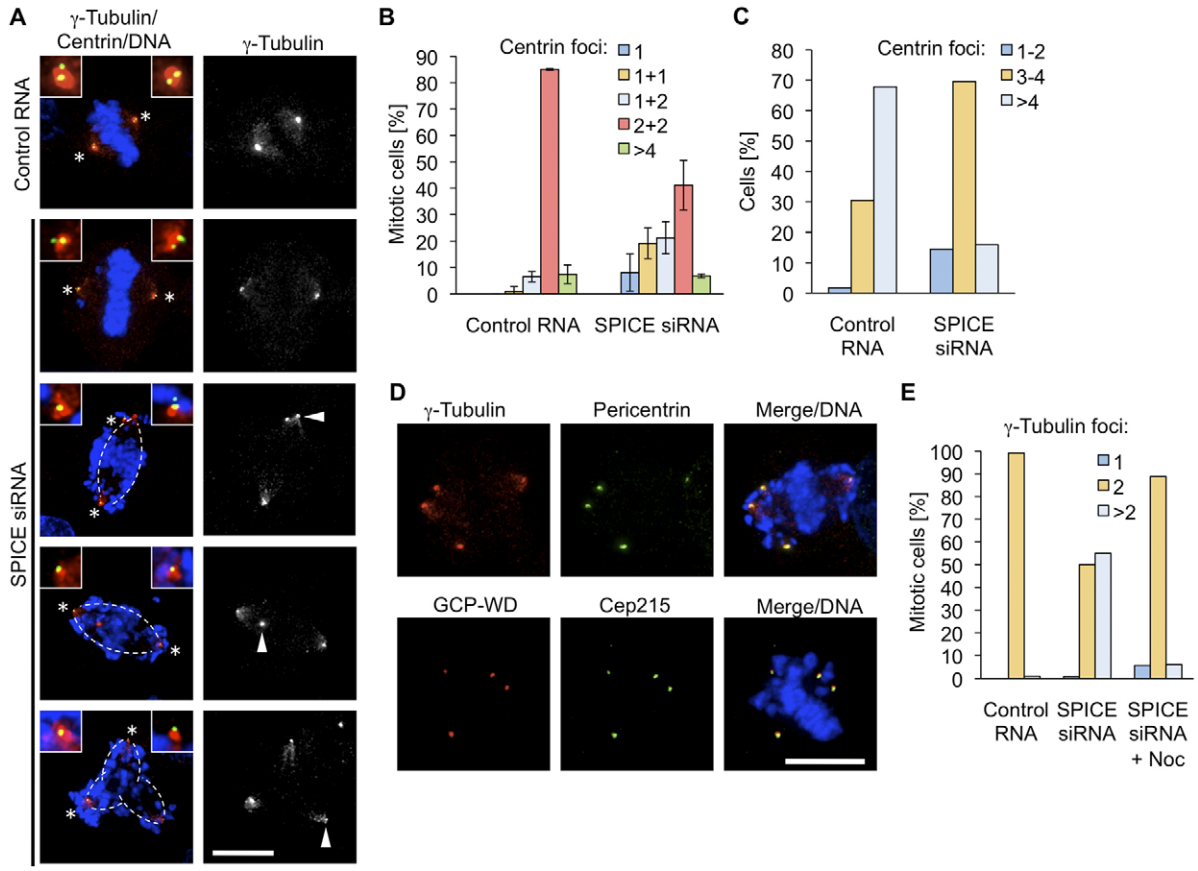

Fig. 3. SPICE is required for centriole duplication. (**A**) Images show maximum projections of deconvolved z-stacks of control and SPICE-depleted HeLa cells stained with anti-centrin antibodies (green), anti-γ-tubulin antibodies (red) and DAPI to visualize DNA. Insets show magnified areas of the two centrosomes, which are labeled with asterisks. The white dashed lines outline the positions of spindles, inferred from foci and diffuse spindle staining of γ-tubulin, and from the orientation of partially aligned chromosomes. Arrowheads mark ectopic, acentriolar γ-tubulin foci. Scale bar: 10 µm. (**B**) HeLa cells were transfected as in A and the centriole configuration in mitotic cells was determined by counting centrin foci (mean of two experiments; >50 cells counted for each sample; error bars indicate s.e.m., 95% CI). (**C**) U2OS cells were transfected with control RNA and *SPICE* siRNA. After 60 hours of S-phase arrest, cells were prepared for immunofluorescence. Centrin foci were counted in >100 cells for each sample. (**D**) Images are maximum projections of deconvolved z-stacks of HeLa cells transfected as in A and stained with antibodies against the indicated proteins. DNA was stained with DAPI. Scale bar: 10 µm. (**E**) HeLa cells transfected as in A and treated with nocodazole as indicated were processed for immunofluorescence. The percentage of mitotic cells with the indicated number of γ-tubulin foci was determined by counting >100 cells for each sample.

cells, HURP staining was restricted to microtubules in the vicinity of the aligned chromosomes and revealed well-organized K-fibers (Fig. 4C). In SPICE-depleted cells, HURP staining was present, but less confined to the region of the spindle equator. HURP was more broadly distributed along the entire spindle, with accumulation near the poles, especially in cells that had only few or no congressed chromosomes (Fig. 4C, bottom panels). Visualization of K-fibers by selective depolymerization of less stable, dynamic spindle microtubules confirmed that SPICE-depleted cells contained K-fibers, but these were poorly organized compared with control cells (Fig. 4D). Together, our data indicate that SPICE depletion disrupts spindle architecture, including K-fiber organization, which interferes with chromosome congression. The partial congression that occurs in some SPICE-depleted cells could be the result of residual SPICE activity or might involve a K-fiber-independent congression mechanism (Cai et al., 2009).

Our work shows that the previously uncharacterized protein SPICE is required for centriole duplication, and for proper bipolar spindle formation and chromosome congression in mitosis. Interestingly, the centrosome protein Cep120 was recently shown to interact with SPICE and to be required for centriole duplication (Hutchins et al., 2010), which might indicate that these proteins cooperate in this process. Importantly, not all SPICE-depleted cells with mitotic defects have a reduced centriole number, suggesting that the mitotic defects are not simply caused by a failure to duplicate centrioles. A more direct role for SPICE in the assembly of proper mitotic spindles would be consistent with the localization of SPICE to both centrioles and spindle microtubules, mediated by different regions within the protein. To our knowledge, a similar localization pattern has been described only for subunits of the augmin complex. However, augmin-depleted cells contain a normal number of centrioles (Lawo et al., 2009), and a role in centriole duplication has not been reported (Dobbelaere et al., 2008).

The core centriole assembly pathway was initially described in *Caenorhabditis elegans* and is conserved in various animal species (Nigg, 2007; Strnad and Gonczy, 2008; Cunha-Ferreira et al., 2009a; Nigg and Raff, 2009). SPICE-related sequences seem to be present only in vertebrates. However, we cannot rule out that SPICE is functionally related to known components of the centriole duplication pathway in other species, many of which have no or

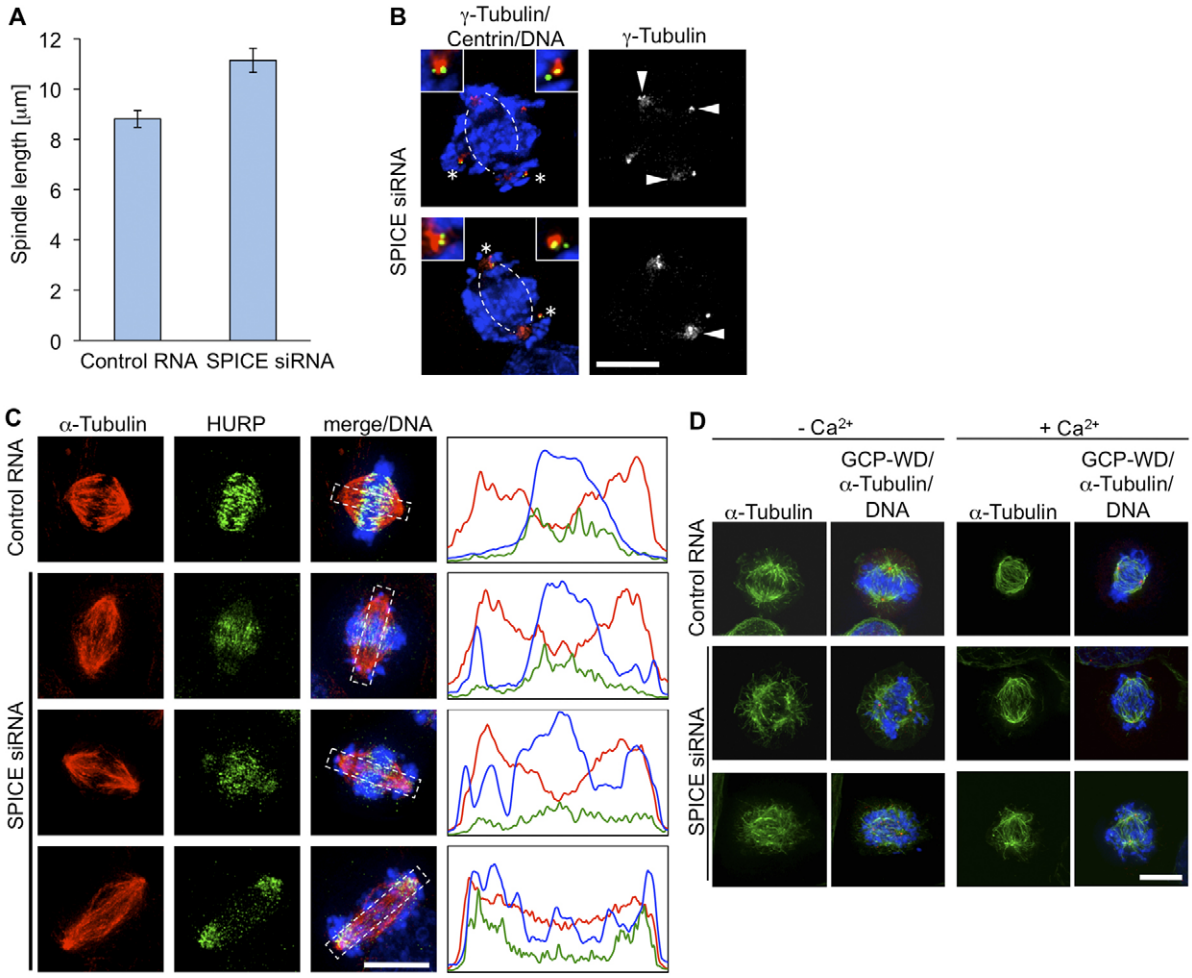

Fig. 4. SPICE depletion impairs spindle architecture independent of centriole number. (**A**) Mean spindle length in HeLa cells transfected with control RNA or *SPICE* siRNA was determined by measuring the pole-to-pole distance in mitotic cells containing two centrin dots at each pole (n>20; error bars indicate s.e.m., 95% CI). (**B**) Cells were transfected, analyzed and projected as in Fig. 3A. Staining of centrin is shown in green, γ-tubulin in red and DNA in blue. Insets show magnified areas of the two centrosomes, which are labeled with asterisks. Spindle orientation, outlined by white dashed lines, was inferred from the foci and diffuse spindle staining of γ-tubulin, and from the orientation of partially aligned chromosomes. Arrowheads indicate acentriolar γ-tubulin foci. (**C**) Images show maximum projections of deconvolved z-stacks of control and SPICE-depleted HeLa cells co-stained with antibodies against α-tubulin and HURP, and with DAPI to visualize DNA. Signal intensities were quantified within a rectangular region along the spindle axis (white dashed lines). The plots show the intensity profiles obtained for microtubules (red), HURP (green) and DNA (blue). Scale bar: 10 µm. (**D**) Control and SPICE-depleted HeLa cells were left untreated (–Ca^{2+}) or extracted with calcium-containing buffer (+Ca^{2+}) to destabilize non-kinetochore spindle microtubules. Cells were fixed and stained with antibodies against α-tubulin and GCP-WD. DNA was stained with DAPI. Shown are maximum projections of deconvolved z-stacks. Scale bar: 10 µm.

little similarity to vertebrate proteins (Dobbelaere et al., 2008). Alternatively, SPICE might have a more specialized function specific to the assembly of vertebrate centrioles.

Materials and Methods

Molecular biology

Full-length SPICE and SPICE fragments were PCR amplified from a cDNA clone (accession number BC036951; Open Biosystems) using the following primers: 5′-TTTGGCCGGCCTATGTCATTTGTCAGAGTGAAC-3′ and 5′-TTTGGCGCGC-CTGATACATGGGTAGAAAGAGC-3′ (full length); 5′-TTTGGCCGGCCTATGT-CATTTGTCAGAGTGAAC-3′ and 5′-TTTGGCGCGCCAATAGCATGTGTGAGT-GATATCAG-3′ (1–450); 5′-TTTGGCCGGCCTATGCGGTGGACAGGTCGCGAG-3′ and 5′-TTTGGCGCGCCCTCTATCAACTGCAGTAGTTTTCC-3′ (350–750); 5′-TTTGGCCGGCCTATGATATCACTCACACATGCTATT-3′ and 5′-TTTGGC-GCGCCTGATACATGGGTAGAAAGAGC-3′ (444–856); 5′-TTTTGGATCCAT-GTCATTTGTCAGAGTGAAC-3′ and 5′-TTTTCTCGAGTCTAAGACGAAGA-ATTTCAGC-3′ (1–422).

For expression in human cells, amplified SPICE sequences were inserted into plasmid pEGFP-C1 (Clontech) containing a modified multiple cloning site using *Fse*I and *Asc*I restriction sites. For expression in *Escherichia coli*, an N-terminal SPICE fragment (amino acids 1–422) was inserted into the pGEX-4T-1 vector (GE Healthcare) using *Bam*HI and *Xho*I restriction sites.

For RNAi-mediated depletion of SPICE, we designed RNA oligonucleotides targeting the following sequences within the SPICE open reading frame: GCUGAGAACAAAUGAGUCA (oligo 1) and GGGAGAUUCACACCUCUUA (oligo 2). For live-cell imaging, we depleted SPICE by expressing a small hairpin RNA (shRNA) that targets the same sequence as oligo 1 (Mission shRNA library; Sigma).

Sequence analysis and alignments were performed with Geneious software (Biomatters). Coiled-coil regions in SPICE were predicted using COILS (Lupas et al., 1991).

Antibodies and reagents

To generate anti-SPICE antibodies, an N-terminal SPICE fragment (amino acids 1–422) was expressed and affinity purified as a soluble GST fusion protein in *E. coli* using glutathione–sepharose (GE Healthcare), according to the manufacturer's standard protocol. The protein was then used for immunization of rabbits (Antibody Production Service, IBMB, CSIC, Barcelona, Spain). After passing the rabbit serum over a resin

bearing immobilized GST to eliminate GST-reactive antibodies, SPICE-specific antibodies were affinity purified using GST-C-SPICE immobilized on Affi-Gel15.

The following additional antibodies were used in this study: rabbit anti-SPICE (sc-102414; Santa Cruz Biotechnology), mouse anti-γ-tubulin (GTU-88; Sigma), rabbit anti-γ-tubulin (Sigma), mouse anti-α-tubulin (DM1A, Sigma), mouse anti-GCP-WD (7D10, Abnova), rabbit anti-pericentrin (Luders et al., 2006), mouse anti-GFP (3E6, Molecular Probes), rabbit anti-GFP (Torrey Pines Biolabs), rabbit anti-Cep215 (IHC-00063, Bethyl Laboratories), rabbit anti-centrin (Groen et al., 2004), rabbit anti-HURP (Wong and Fang, 2006), rabbit anti-p38 MAP kinase (Santa Cruz Biotechnology), mouse anti-CENP-E (Rockland Immunochemicals) and human CREST autoantibody (Fitzgerald Industries International).

Alexa-Fluor-488- and Alexa-Fluor-568-conjugated secondary antibodies used for immunofluorescence microscopy were from Invitrogen. Peroxidase-coupled secondary antibodies for western blotting were from Jackson ImmunoResearch Laboratories.

Cell culture, transfection and drug treatments

HeLa and U2OS cell lines were grown in DMEM containing 10% FCS.

Cells were transfected with plasmid or small interfering RNA (siRNA) using Lipofectamine 2000 or Lipofectamine RNAiMAX (Invitrogen), respectively.

For the centriole overduplication assay, U2OS cells grown on coverslips were transfected with siRNA targeting SPICE and arrested in S phase after 24 hours by the addition of 4 mM hydroxyurea (Sigma). After 60 hours, cells were fixed and processed for immunofluorescence.

To depolymerize microtubules, cells were treated with 2.5 μg/ml nocodazole 48 hours after siRNA transfection and incubated for 2.5 hours at 37°C.

To selectively destabilize non-kinetochore microtubules, HeLa cells were treated in destabilization buffer (100 mM PIPES pH 6.8, 0.2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% Triton X-100) for 2 minutes at room temperature.

Western blotting

Cells were washed in PBS and lysed (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5% NP-40, 1 mM DTT, protease inhibitors) on ice. Cleared extracts were prepared by centrifugation and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes and probed with antibodies.

Fluorescence microscopy

HeLa cells grown on coverslips were fixed in methanol at –20°C for at least 5 minutes and processed for immunofluorescence. Alternatively, for K-fiber visualization, cells were fixed with 3.7% formaldehyde in destabilization buffer for 20 minutes at room temperature. Fixed cells were blocked in PBS-BT (1× PBS, 0.1% Triton X-100 and 3% BSA) and incubated with antibodies in the same buffer. Images were acquired with an Orca AG camera (Hamamatsu) on a Leica DMI6000B microscope equipped with 1.4 NA 63× and 100× oil immersion objectives. AF6000 software (Leica) was used for image acquisition and the deconvolution and maximum projection of z-stack images. For further image processing and quantification of fluorescence intensities, ImageJ software was used. Intensities were measured in images acquired with constant exposure settings and were background corrected.

For time-lapse microscopy, HeLa cells were co-transfected 48 hours before image acquisition with H2B–mCherry (Addgene) and with a vector expressing SPICE shRNA (Mission shRNA; Sigma) or with a control vector. An automated Olympus IX81 microscope equipped with a temperature-controlled CO_2 incubation chamber and a 20× 0.45 phase-contrast objective was used for time-lapse imaging. Images were acquired every 10 minutes for 24 hours with an ORCA camera (Hamamatsu). Subsequent analysis was performed with ImageJ software.

We are grateful to Aaron Groen and Ryoma Ohi for the kind gift of the anti-centrin antibody, and to Jim Wong and Guowei Fang for the anti-HURP antibody. We thank Joan Roig and Travis Stracker for fruitful discussions and critical reading of the manuscript, and Maria Teresa Bertran for help with time-lapse imaging. J.L. acknowledges support from Plan Nacional grant BFU2009-08522 (Ministerio de Ciencia e Innovación, Spain), from Marie Curie International Re-integration Grant FP7-PEOPLE-2007-4-3-IRG #224835 (European Commission) and from IRB Barcelona intramural funds.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/18/3039/DC1

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