ATPase SRCAP is a new player in cell division, uncovering molecular aspects of Floating-Harbor syndrome

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Abstract

Floating-Harbor syndrome (FHS) is a rare inherited developmental disorder characterized by bone mineralization delay and growth deficits frequently associated with skeletal and craniofacial defects and mental retardation. The major cause of FHS is heterozygous truncating mutations in *Srcap*, which encodes the ATPase SRCAP, the core catalytic subunit of the homonymous multiprotein chromatin-remodeling complex in humans. This complex promotes the exchange of canonical histone H2A with the H2A.Z variant. SRCAP is also implicated in several cellular processes, but the molecular basis of FHS still needs to be elucidated. Using a combined approach, we studied the involvement of SRCAP in cell cycle progression in HeLa cells. In addition to the canonical localization in interphase nuclei, both SRCAP and its *Drosophila* orthologue localized to the mitotic apparatus after nuclear envelope breakdown, and their depletion impaired mitosis progression and cytokinesis in human and Drosophila cells, respectively. Importantly, SRCAP interacted with cytokinesis regulators and α-Tubulin during telophase, strongly supporting a direct role in cytokinesis, independent of its chromatin remodeling functions. Our results provide clues about previously undetected, evolutionarily conserved roles of SRCAP in ensuring proper mitosis and cytokinesis. We propose that perturbations in cell division contribute to the onset of developmental defects characteristic of FHS.

Introduction

In the last few decades, a large body of experimental evidence has suggested that mutations in genes encoding a variety of chromatin factors and epigenetic regulators, such as DNA or histone modifying enzymes and members of ATP-dependent chromatin remodeling complexes, are crucial players in human genetic diseases and cancer (1-7). Floating-Harbor syndrome (FHS), also known as Pelletier–Leisti syndrome, is a human developmental disorder characterized by delayed bone mineralization and growth deficiency, which are often associated with mental retardation and skeletal and craniofacial abnormalities (8-11).

Srcap (SNF2-Related CBP Activator Protein) is the causative gene of the FHS. It maps to chromosome 16p11.2 and encodes the ATPase SRCAP, the core catalytic subunit of the homonymous multiprotein chromatin-remodeling complex in humans. The SRCAP complex is member of the evolutionarily conserved INO80 family of ATP-dependent chromatin remodeling complexes (12, 13) and contains a dozen subunits (12-18). The primary function of the SRCAP complex is to catalyze the incorporation of H2AZ-H2B dimers into nucleosomes (19, 20).

FHS has a dominant inheritance pattern caused by nonsense or frameshift mutations in exons 33 and 34 of *Srcap*. (9, 11). These mutations are supposed to produce a C-terminal-truncated SRCAP protein variant missing the AT-hook motifs with DNA-binding activity and are possibly responsible for a dominant negative effect (9, 11) triggering the onset of FHS. Recently, localization assays in human embryonic cranial neural crest cells showed that overexpressed GFP/Flag-tagged versions of C-terminal-truncated SRCAP are largely excluded from the nucleus but present in the cytoplasm (21), suggesting that FHS mutations affect the nuclear localization of SRCAP. The SRCAP protein can also function as a transcriptional activator by binding to the cAMP response element-binding protein (CREB)-binding protein (CREBBP or CBP) (22). Moreover, a role of SRCAP in DNA-end resection was proposed

recently (23).

DOMINO-A (DOM-A), the functional SRCAP orthologue in *Drosophila melanogaster* (24, 25), is the main subunit of the *Drosophila* Tip60 (dTip60) chromatin remodeling complex (26). The dTip60 subunits share high sequence identity and functional conservation with SRCAP and p400/Tip60 human complexes (12).

Overall, SRCAP appears to be a multifaceted protein implicated in several cellular processes, including chromatin regulation, transcription, and DNA repair (12, 19, 20, 22, 23, 27). Therefore, investigating the wild-type cellular functions of SRCAP may provide clues to the genetic and molecular basis of FHS onset (11).

We combined cell biology, functional genomics, and biochemical approaches to study the subcellular distribution of the SRCAP protein and assess its involvement in cell cycle progression in HeLa cells. Unexpectedly, we found that SRCAP associates with components of the mitotic apparatus, including centrosomes, the spindle, and midbody, and interacts with a plethora of cytokinesis regulators. Furthermore, its RNAi-mediated depletion perturbs mitosis and cytokinesis. Similarly, DOM-A localizes to centrosomes and the midbody, and its depletion results in cell division defects in Drosophila S2 cells. Our findings suggest that, in addition to the established role as epigenetic regulator, SRCAP plays previously undetected and evolutionarily conserved roles in cell division. Moreover, our results emphasize a surprising scenario whereby alterations in cell division produced by SRCAP mutations are important causative factors co-occurring at the onset of FHS.

Results

SRCAP is recruited to the mitotic apparatus in human cell lines

First, we investigated the subcellular distribution of endogenous SRCAP protein during the cell cycle in HeLa cells using immunofluorescence microscopy (IFM). A specific polyclonal

antibody decorated the interphase nuclei, as expected, but also revealed a specific pattern at the mitotic apparatus during mitotic progression. Soon after nuclear envelope breakdown, SCARP-specific immunofluorescence redistributed at the mitotic spindle with enrichment at the poles and centrosomes, and later at the central spindle and midbody (Figure 1). These observations were recapitulated in the HuH7 hepatocyte carcinoma-derived cell line (28) and in human MRC5 fibroblast-derived cell line. As shown in Supplemental Figure 1, immunofluorescent signals were observed at both the spindle and midbody, in line with the results in HeLa cells (Figure 1).

The specificity of the SRCAP antibody was validated by Western blotting and IFM performed after RNAi-mediated depletion of SRCAP in HeLa cells (Supplemental Figure 2). On Western blots, the SRCAP band detected in mock-treated cells was absent from RNAi-treated cells (Supplemental Figure 2A). Similarly, the immunofluorescence decreased in RNAi-treated HeLa cells compared to mock-treated controls (Supplemental Figure 2, B and C). Taken together, these results indicate that the observed localization reflects intrinsic properties of the SRCAP protein, with no cell type specificity.

The midbody is a tightly packed bridge that forms from the bipolar microtubule array derived from the anaphase central spindle. It serves as a platform for orchestrating cytokinesis by recruiting a large number of factors needed for abscission, the last stage of cell division (29). Therefore, we wanted to evaluate the midbody association of SRCAP using both IFM and Western blotting on isolated midbodies (see Methods). As shown in Figure 2A, SRCAP immunofluorescence decorated the isolated midbodies. Notably, this midbody localization was dynamic, as the fluorescence was more widely distributed in early telophase and became gradually more restricted in late telophase. Such behavior is similar to that of other known cytokinesis regulators (30). Western blot analysis of protein extracts from isolated midbodies detected two SRCAP bands of approximately 350 and 300 KDa (Figure 2B).

Taken together, these findings show that the subcellular localization of SRCAP is dynamic during cell division and it is recruited not only to interphase nuclei, but also to the centrosomes, spindle, and midbody. Remarkably, SRCAP is the core subunit of the homonymous complex governing H2A.Z deposition into chromatin (12, 19, 20); thus, its association with the mitotic apparatus was not obvious.

SRCAP protein depletion perturbs mitosis and cytokinesis in HeLa cells

Next, we examined the functional significance of SRCAP recruitment to centrosomes, the spindle, and midbody during cell division by investigating the progression of cell division in SRCAP-depleted HeLa cells utilizing RNAi-mediated silencing. In fixed HeLa cell preparations, we categorized and quantified five classes of cell division defects (Figure 3, A-G, Table 1): multipolar spindles (MS) at pro-metaphase and metaphase (Figure 3B), chromosome misalignments (CM) at metaphase (Figure 3C), chromatin bridges (CB) at anaphase and telophase (Figure 3D), long thin intercellular bridges (LIB) at the last stage of telophase (Figure 3E), and multinucleated cells (MC) (Figure 3F). Compared to mock-treated control cells, SRCAP RNAi-treated cells exhibited a significant increase in mitosis and cytokinesis defects in all classes except MS. The increase was particularly relevant for CM (57%) and LIB (29%). A LIB is defined as overextended, stretched, intercellular bridge that forms as a consequence of a failure of abscission, the final stage of cytokinesis. Consistently, the intercellular distance at the abscission stage in SRCAP-depleted cells was also increased compared to control cells (Figure 3G). Notably, no trapped chromatin was observed in the observed LIB. Defective cytokinesis was also reflected in the appearance of MC (14%), whereas the formation of CB was mild compared to the mock-treated control. Moreover, in agreement with defective mitosis, SRCAP-depleted cells exhibited a significant amount of altered spindles with a shorter and thinner shape (ASM) than those in the control cells (Figure

3C). Thus, it appears that SRCAP depletion disrupts both mitosis and cytokinesis in HeLa cells, suggesting that the localization observed at centrosomes, the spindle, and midbody reflects its functional roles in cell division.

Microtubule re-polymerization is affected in SRCAP-depleted cells

The finding that SRCAP depletion affects spindle morphology and chromosome alignment at metaphase (Figure 3C) suggests that this protein regulates microtubule organization and mitotic spindle assembly. To test this hypothesis, we used HeLa cells stably expressing EGFP::α-Tubulin and assessed whether the presence or absence of SRCAP influences microtubule regrowth after cold-induced disassembly. Control (mock) and SRCAP RNAi-depleted HeLa cells (RNAi) were incubated on ice for 1 hour to induce extensive depolymerization (T0). The cells were then allowed to rewarm at 37°C in complete medium for 5 min (T5) to resume microtubule regrowth. As shown in Figure 4, microtubule re-polymerization after 5 minutes of rewarming resulted in clearly aberrant asters with rare, long, and thin MTs in RNA-treated cells (mean±SD, 36.84% ± 3.76) compared to the mock-treated cells (mean±SD 5% ± 1.92). This result supports a role of the SRCAP protein in microtubule organization and mitotic spindle assembly.

SRCAP depletion affects the localization of cytokinesis regulators to the midbody in HeLa cells

Cytokinesis is the last step of cell division and is controlled by a plethora of essential regulators recruited to the midbody during telophase (29-35). The finding that SRCAP-depleted cultures are enriched in LIB and MC (Figure 3) prompted us to investigate a possible role of SRCAP in cytokinesis. We used IFM to study the recruitment of crucial regulators of cytokinesis to the midbody in HeLa cells proficient or depleted in SRCAP. We focused on

CIT-K, MKLP2, Aurora B, INCENP, MKLP1, PLK1, CEP55, Anillin, Alix, and Spastin, nine well-known proteins that localize to the midbody and are required for cytokinesis (29-35). The results of three independent replicates indicate that the midbody localization of these molecules was impaired, with the exception of CIT-K (Figure 5). For example, the midbody localization of Aurora B and Anillin was severely affected, while that of PLK1 became more widely distributed.

SRCAP interacts with cytokinesis regulators

The results of the experiments carried out thus far suggest that the recruitment of a number of cytokinesis regulators to the midbody depends on SRCAP activity. We performed a series of simultaneous IFM experiments to evaluate whether SRCAP co-localizes at the midbody with the above tested regulators of cytokinesis (Figure 5). Remarkably, SRCAP co-localized at the midbody with MKLP1, MKLP2, Aurora B, INCENP, CEP55, Anillin, Alix, and Spastin, but only partial co-localization was observed with CIT-K (Figure 6).

The results of these experiments are suggestive of possible interactions between SRCAP and the tested regulators of cytokinesis. To test this hypothesis, we carried out immunoprecipitation (IP) assays on protein extracts from telophase-synchronized HeLa cells, using a SRCAP antibody by Kerafast (Supplemental Figure 3 and Ref 14). Synchronization was followed by subcellular fractionation assays to recover the cytoplasmic component (S2 fraction) and segregate away the chromatin-associated components (Figure 7, A and B; see Methods). Figure 7C shows the comparison between the IP (+SRCAP) and negative control (-SRCAP). SRCAP appears to interact with CIT-K, MKLP2, Aurora B, PLK1, CEP55, Anillin, Alix, and Spastin in telophase. Moreover, we found that SRCAP interacts with α-Tubulin, the main structural component of the midbody. The IP experiments did not reveal any SRCAP interactions with MKLP1 and INCENP (not shown).

Inhibition of Aurora B kinase activity does not affect SRCAP recruitment to the midbody

Our results have shown that SRCAP interacts with Aurora B and contributes to its recruitment to the midbody (Figures 5, 6 and 7). Aurora B is a key regulator of cell division, and its kinase activity promotes the recruitment of several motor and microtubule-associated proteins in the last stages of cell division and controls abscission when the midbody must is severed (36-38). To gain clues about the factors controlling SRCAP recruitment to the midbody, we treated HeLa cells with ZM447439, a specific selective inhibitor of Aurora B kinase activity (39). The ZM447439 treatment impaired the localization of MKLP1 at the midbody (Figure 8 and Supplemental Table 3), a well-known target of Aurora B phosphorylation during the final stage of cell division (40). In contrast, the localization of SRCAP was not significantly affected, or that of Aurora B. This result indicates that, although Aurora B interacts with SRCAP by IP, its kinase activity is not responsible for the recruitment of SRCAP to the midbody.

Localization and RNAi-mediated depletion of DOM-A in Drosophila S2 cells

Lastly, we investigated whether the association of SRCAP with the mitotic apparatus and defects in cell division observed after its depletion are unique to human cells or are evolutionarily conserved. First, we used IFM to study the localization of DOM-A, the *Drosophila* ortholog of human SRCAP, in *Drosophila melanogaster* S2 cells. In addition to the interphase nuclei, a DOM-A antibody (24) decorated centrosomes and the midbody (Figure 9). Next, we examined the phenotypes of S2 cells after RNAi against DOM-A. The RNAi efficiency was tested by immunofluorescent assays and sqRT-PCR (Supplemental Figure 4), because the DOM-A antibody did not work properly for Western blotting under our conditions. Depletion of DOM-A resulted in mitotic phenotypes comparable to those observed

in SRCAP-depleted HeLa cells (Figure 10 and Table 4). Five categories of significant defects were observed: MS (46%), CMs (21%), CB (4%), LIB (19%), and MC (12%). Importantly, these defects are consistent with the localization of DOM-A to centrosomes and the midbody in S2 cells.

Next, stained DOM-A-depleted S2 cells with an antibody against Spd2, a Drosophila centriole protein used as a centrosome marker (41). We observed a high percentage of metaphase with multiple centrosomes exhibiting MT-nucleation ability, which gives rise to MSs (Supplemental Figure 5). Multiple centrosomes may arise from aberrant centriole disengagement/amplification, which in turn leads to the formation of MS and chromosome mis-segregation (42). Alternatively, abnormal numbers of centrosomes can be a consequence of cytokinesis failure that results in the formation of MC.

Discussion

The ATPase SRCAP is the core subunit of the SRCAP complex and performs crucial roles in chromatin organization and transcriptional regulation. Here, we demonstrated that SRCAP relocates to centrosomes, the spindle, and midbody during cell cycle progression, with its depletion yielding an array of aberrant outcomes of mitosis and cytokinesis. Similarly, DOM-A is found at centrosomes and the midbody in *Drosophila* S2 cells, and its depletion affects both mitosis and cytokinesis (compare Figures 9 and 10). Notably, defects in cell division resembling those found after SRCAP and DOM-A depletion occur as a consequence of the loss of crucial regulators of spindle and/or midbody organization or function (29-35).

How does depletion of SRCAP and DOM-A cause defects in mitosis and cytokinesis?

At least two alternative hypotheses can be considered to explain the defects found after SRCAP and DOM-A depletion. First, a lack SRCAP or DOM-A may give rise to aberrant

chromatin changes that alter the expression of genes involved in cell division and/or to perturbations in kinetochore and spindle organization and function. According to this hypothesis, the cell division defects caused by SRCAP and DOM-A depletion are indirect, and the recruitment of SRCAP or DOM-A to the mitotic apparatus only reflects a passive accumulation of disposable factors. Second, SRCAP and DOM-A proteins are essential components of the mitotic apparatus and regulate cell division independent of their functions in chromatin regulation. If this is true, SRCAP and DOM-A depletion directly affects mitosis and cytokinesis.

Several lines of evidence favor the last hypothesis. First, the recruitment of SRCAP and DOM-A to the mitotic apparatus, together with the disruption of specific steps of mitosis and cytokinesis caused by their depletion in humans and *D. Melanogaster,* respectively (organisms separated by about 800 myr), are evolutionarily conserved phenomena. Second, the observed defects do not appear to simply be a chaotic disruption of cell division as one would expect by simultaneous up- or down-regulation of a group of genes encoding regulators of cell division due to epigenetic alterations. In contrast, SRCAP and DOM-A depletion yields to an array of aberrant mitotic outcomes falling into specific categories of alterations in mitosis and cytokinesis. Notably, such alterations are consistent with the localization of SRCAP and DOM-A proteins to the mitotic apparatus and also occur after the loss of key regulators of mitosis and cytokinesis (29-35). Finally, and most importantly, a direct role of SRCAP in the final stage of cell division, independent from its chromatin functions, is primarily supported by the recruitment of MKLP2, Aurora B, PLK1, CEP55, Anillin, Alix, and Spastin to the midbody being affected by SRCAP depletion and the same proteins co-localizing with SRCAP at the midbody (Figures 5 and 6). These proteins also interact with SRCAP in co-IP assays of chromatin-free protein extracts from the cytoplasmic fraction of telophase-synchronized HeLa cells (Figure 7). Notably, the interaction between SRCAP and Anillin has

also been highlighted in a recent study on the midbody interactome (43).

All of the proteins identified here as SRCAP interactors in telophase are essential for successful cell division in different organisms, as their depletion results in aberrant cytokinesis. CIT-K is the main abscission regulator capable of physically and functionally interacting with the actin-binding protein Anillin, a crucial component of the contractile ring and midbody (29, 44, 45); MKLP2 is a motor kinesin that binds microtubules and is required for Aurora B recruitment at the central spindle (29, 46). CEP55 recruits Alix at the midbody. Notably, in the absence of CEP55, a series of late-acting abscission factors fail to concentrate at the midbody, including Aurora B, MKLP2, Plk1, PRC1, and ECT2 (47), and the ESCRT machinery. Spastin is a key player in microtubule severing, ensuring the final cut at the midbody, whereas α-Tubulin is a major component of spindle and midbody microtubules.

Although we cannot completely rule out a contribution of chromatin perturbations in cell division defects, our results suggest that SRCAP and DOM-A are multifaceted proteins that, in addition to their canonical functions in interphase, may play conserved functions in mitosis and cytokinesis. Sparse experimental evidence in the literature also provides evidence for specific roles of chromatin remodeling factors in cell division that are distinct from their functions in chromatin regulation (48-56).

What are the molecular functions of SRCAP?

Although our data suggest that SRCAP has direct roles in cell division, its molecular functions are not well-defined. Spindle reformation defects, cell division alterations, and mis-localization of cytokinesis regulators at the midbody in SRCAP-depleted cells, together with specific interactions detected in telophase-synchronized cells provides evidence that SRCAP participates in two different steps of cell division; it may ensure proper chromosome segregation, regulating microtubule organization and mitotic spindle assembly, and it may be

required for midbody function during abscission, participating in the recruitment of cytokinesis regulators, such as MKLP2, Aurora B, PLK1, Cep55, Anillin, Alix, and Spastin (and possibly other factors) to the midbody, ensuring the final cut that is essential for proper abscission. In this context, crosstalk between SRCAP and cytokinesis regulators may occur to control midbody architecture and function similar to the examples in the literature (38).

Several ATPases, such as Katanin, Cdc48/p97, ISWI, VPS4, and Spastin, interact with microtubules and play direct roles in mitosis and cytokinesis (57-59). The ESCRT-III complex protein CHMP1B recruits Spastin to the midbody (58). Intriguingly, depletion of Spastin results in cytokinesis failure phenotypes similar to those found in SRCAP-depleted cells (60). Thus, the ATPase activity of SRCAP may also be required for its function in mitosis and cytokinesis. In particular, during the final stage of cell division, SRCAP may act as a platform to recruit/stabilize key cytokinesis/abscission regulators to the midbody to ensure the final cut. The precise mechanism underlying the role of SRCAP in cell division will be an important topic for further analysis.

In conclusion, our results highlight previously undetected and evolutionarily conserved roles of SRCAP in ensuring faithful cell division. Therefore, we propose that mitosis and cytokinesis failure are important causative factors co-occurring at the onset of developmental defects characteristic of FHS. It is well known that defective mitosis or cytokinesis can cause chromosomal instability leading to genetically unstable states, hence activating tumorigenic transformation (61-62). A first case of a tumor associated with FHS has indeed been reported in 2009 (63).Thus, it might be possible that FHS patients also exhibit some predisposition for tumor development. If this was true, than the FHS patients should be subjected to clinical trials for cancer prevention.

Methods

Cell Cultures, transfections and RNAi treatments. HeLa cells, purchased from ATTC company, were cultured in 6-well plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Corning) and a penicillin/streptomycin solution (Gibco, 15140122). Double transfection (24h + 48h after seeding) was performed with siRNA targeting SRCAP transcripts (sc-93293, Santa Cruz), using Lipofectamine RNAiMAX transfection reagent (Thermo Scientific), according to the manufacturer's protocol. 24h after the second transfection cells were harvested for cytological and immunoblotting analysis.

Drosophila melanogaster S2 cells were cultured at 25°C in Schneider's *Drosophila* Medium (Biowest) supplemented with 10% heat-inactivated FBS, 50 units/ml penicillin and 50 µg/ml streptomycin in 75-cm^2 T-flasks. RNAi treatments were carried out according to Clemens et al. (2000). Cells were suspended in serum-free Schneider's medium at a concentration of 1×10^6 cells/ml and plated 1 ml/well in a six-wells culture dish. To perform DOM-A RNAi, each culture was inoculated with 15 µg of dsRNA. After 1h incubation at 25°C, 2 ml of complete medium were added to each well. Control cultures were prepared in the same way without addition of dsRNA. Both dsRNA-treated and control cells were grown for 96h at 25°C and then processed for either immunofluorescence or blotting analysis. To prepare dsRNA, individual gene sequences were amplified by PCR from genomic DNA obtained from first-instar larvae of a wild type *D. melanogaster* strain. The primers used in the PCR reactions were 48 nt base long and all contained a 5' T7 RNA polymerase binding site (5'-GAATTAATACGACTCACTATAGGGAGAC-3') joined to a DOM-A specific sequence. The sense and antisense gene-specific DOM-A primers were as follows: for-TCTGGTGCTCAGATCGTGTC; rev-GTTGTCTGCAGCACCTTCAA.

Cytology and immunostaining. For immunofluorescence (IF) staining, HeLa cells were seeded on glass coverslips and 24h later they were fixed for 15′ at room temperature (RT) in 2% formaldehyde in PBS or for 2’ in cold-methanol. After permeabilization in 0.2% Triton X-100 solution (5’ in ice) and washing in PBS, the cells were incubated in 3% bovine serum albumin (BSA) for 1h and, subsequently, over-night (O/N) with primary antibodies (Supplemental Table 1) at 4°C. After several washes in PBS, cells were incubated with secondary antibodies for 1h at RT. Coverslips were mounted onto slides with ProLong Antifade Mountants for Fixed Cells (Thermo Scientific). Simultaneous IF experiments (Figure 6) were performed using the aforementioned protocol, using a first double incubation with primary antibodies against SRCAP and a protein of interest (Supplemental Table 1), followed by double incubation with secondary antibodies (Supplemental Table 2).

Drosophila melanogaster S2 cells were seeded on glass coverslips and 24h later they were fixed for 20′ at RT in 4% formaldehyde in PBS or 10’ methanol on ice. Then they were permeabilized for 20’ at RT in 0.1% Triton X-100 solution. IF staining was performed using the protocol described before. Both human and *Drosophila melanogaster* slides were analyzed using a computer-controlled Nikon Eclipse 50i epifluorescence microscope equipped with a CCD camera. Fluorescence intensity was assessed using the ImageJ software (http://rsbweb.nih.gov/ij/). Primary and secondary antibodies are listed in Supplemental Tables 1 and 2.

Microtubules re-polymerization assays. HeLa Kyoto EGFP-α-Tubulin/H2B-mCherry cell line was purchased from CLC (EMBL, Germany). Cell cultures and transfection were performed according to the previous section. 24h later last transfection, cells were assayed for microtubules re-polymerization. In this assay, control (Mock) and SRCAP RNAi-depleted

cells (RNAi) were incubated 1h in ice (T0) and then supplemented with complete medium for 5' (T5) to resume microtubules polymerization at 37°C. Asters length was evaluated for analysis using the ImageJ software.

Western blotting. HeLa cells were lysated in sample buffer at 10000 cells/µl. All the samples were load in a polyacrilamyde gel, transferred onto PVDF membrane and probed with specific primary antibodies (Supplemental Table 1) and with appropriate HRP-conjugated secondary antibodies (Supplemental Table 2). The bands were immunodetected using the Enhanced ChemiLuminescence (ECL) kit from Thermo Scientific.

Inhibition of Aurora B kinase activity. HeLa cells were treated for 35' with ZM447439 Aurora B inhibitor at a final concentration of 5 µM and then fixed and stained. An equal volume of DMSO was used as negative control.

Midbody isolation. The midbody association of SRCAP were evaluated on isolated midbodies. Midbody isolation was performed according to McKenzie et al., (38). IFM and Western blotting were performed as described in the above paragraphs.

Cell cycle synchronization and subcellular fractionation assay. For immunoprecipitation experiments, HeLa cells were synchronized in telophase using thymidine/nocodazole blocks. Cells were treated with 2 mM thymidine (Sigma, T9250) for 19h, released from G1/S block in fresh media for 5h, incubated with 40 nM nocodazole (Sigma, M1403) for 13h and harvested by mitotic shake-off. Mitotic cells were washed three times with PBS and released in fresh medium for 70' before harvesting and freezing in liquid nitrogen. Telophase cells (2 x 10^7) were prepared by resuspending in 1 mL of Buffer A (10 mM HEPES at pH 7.9, 10 mM KCl,

1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM dithiothreitol [DTT], 1 mM $NaVO_4$, 5 mM β-glycero-phosphate, 0.1 mM phenyl methane sulphonyl fluoride [PMSF], 0.5 mM NaF, protease inhibitor cocktail [Roche]) for subcellular fractionation assay. Triton X-100 was added at 0.1% and cells were incubated for 5' on ice. After centrifugation (3500 rpm for 5' at 4°C), the cytosolic fraction (supernatant) was used for immunoprecipitation experiments and the nuclear fraction (pellet) was resuspended in 1 mL of Buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 0.1 mM PMSF, protease inhibitors) and incubated for 5' on ice. All the fractioned samples were load in a polyacrylamide gel and transferred onto PVDF membrane for western blot analysis.

Immunoprecipitation. Cytosolic fraction (2 mg/ml) from subcellular fractionation assay was used as input (IN) to immunoprecipitate SRCAP protein using 10 µg of rabbit anti-SRCAP antibody by Kerafast (13) O/N at 4°C, followed by 2h of incubation with 40 µl of agarose-conjugated protein A/G beads (Santa Cruz Biotechnology) on rotating wheel. As negative control, no antibody was added to a same amount of IN and beads. Finally, beads were washed three times in buffer A and co-immunoprecipitants were analyzed by immunoblotting.

Statistical analysis. Data analyses were performed using the GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). All results are expressed as mean ±SD values from three independent replicate experiments. P value of less than 0.05 (*$P < 0.05$, compared with the control group) are considered to be statistically significant by using two-tailed Fisher's exact test.

Author contributions

PD and GM conceptualized and supervised the study; PD wrote the original draft; GM, YP, FDM, MVS and MTA performed the experiments.

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FIGURES AND FIGURE LEGENDS

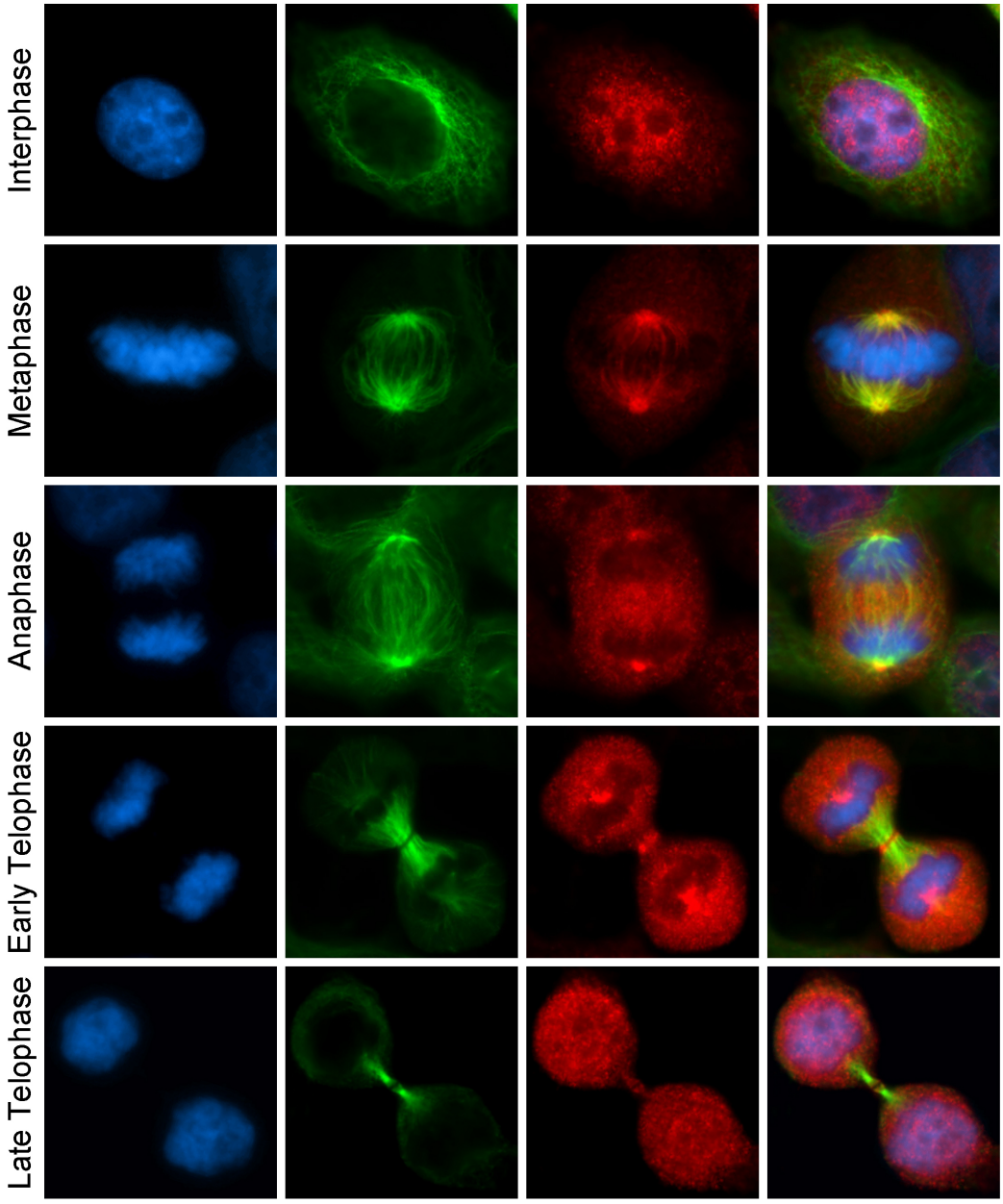

Figure 1. SRCAP localizes to the centrosomes, spindle and midbody in HeLa cells**.**
Fixed HeLa cells stained with DAPI (blue), anti-SRCAP (red) and anti-α-Tubulin (green). As expected, the SRCAP staining is clearly present in the interphase nuclei. At metaphase, the SRCAP staining is found on spindle poles and spindle fibers, while in later stage is found on centrosomes and central spindle (anaphase) and midbody (telophase).

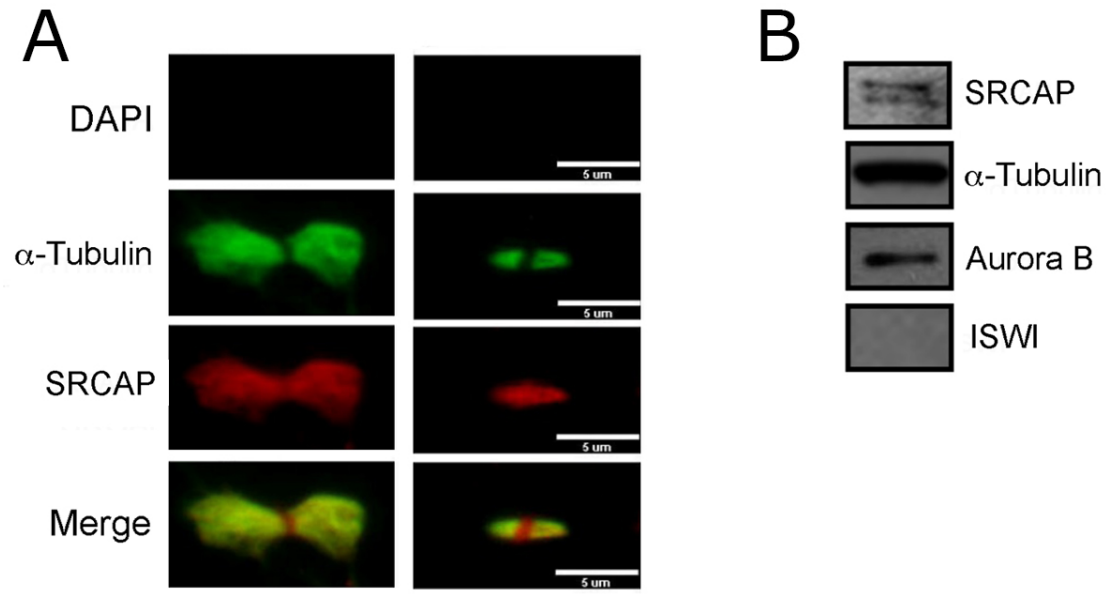

Figure 2. Localization of SRCAP on isolated midbodies**.**

The midbody association of SRCAP was evaluated on isolated midbodies. A) Immunolocalization of SRCAP protein to isolated midbodies. Fixed preparations of isolated midbodies were stained with DAPI (blue), anti-SRCAP (red) and anti-α-Tubulin (green). No DAPI staining was detected. SRCAP staining clearly decorated the isolated midbodies and overlaps with that of α-Tubulin. B) Detection of SRCAP by Western blotting on midbody extracts from HeLa cells. Two SRCAP bands were detected of about 350 and 300 KDa. Aurora B was used as a positive control. The ISWI remodeller was not detected (negative control).

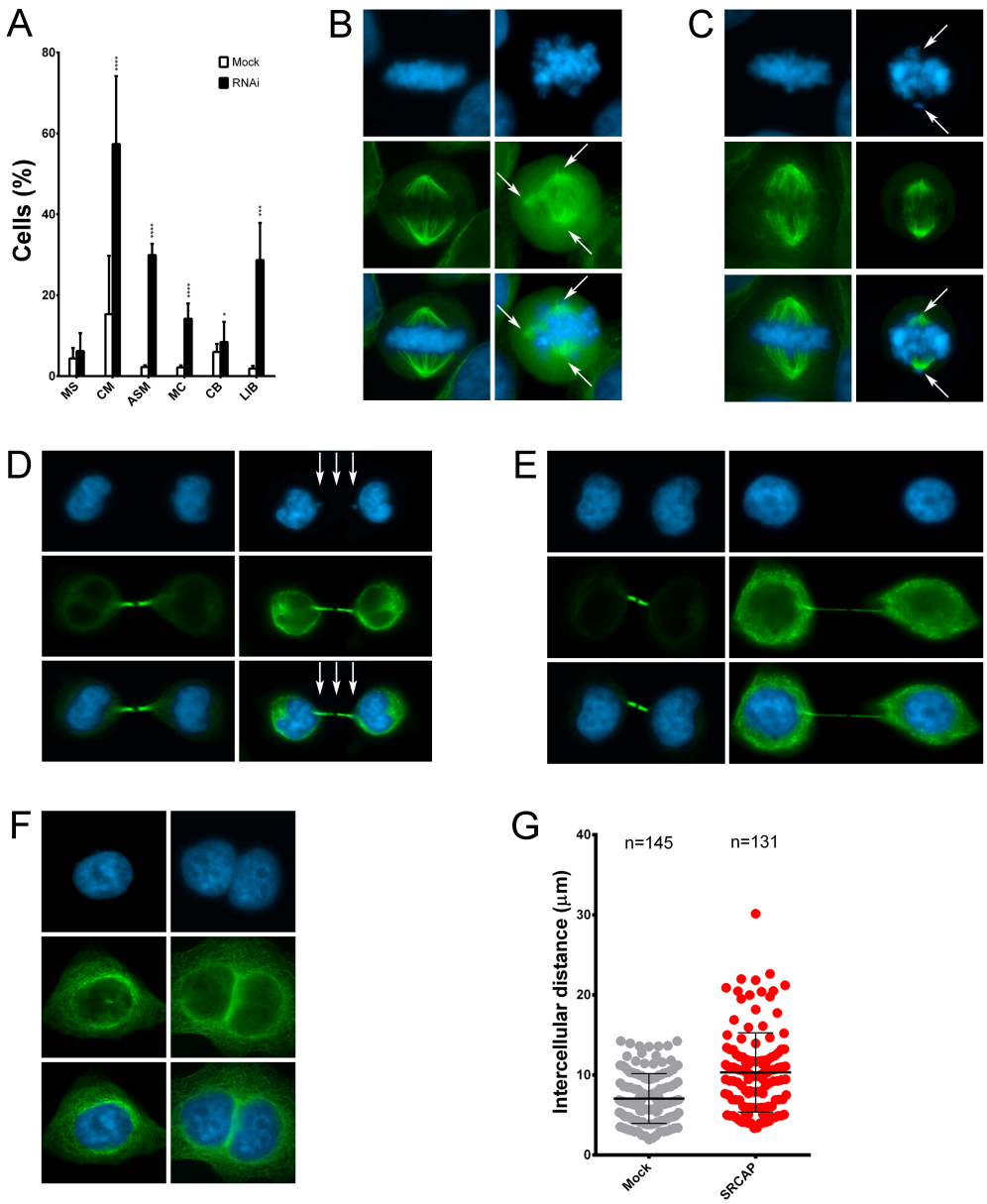

Figure 3. RNAi mediated depletion of SRCAP affects cell division in HeLa cells**.**

RNAi knock-down-mediated experiments were performed by transfecting HeLa cells with specific siRNAs (see Methods). Hela cells were stained with DAPI (blue) and anti-α-Tubulin (green). Five classes of defects were categorized: A) Histograms showing the quantitative analysis of cell division defects B) multipolar spindles (MS); C) chromosome misalignments (CM); D) chromatin bridges (CB); E) long intercellular bridges (LIB); F) multinucleated cells (MC); G) Intercellular distance. ASM = Altered spindles morphology. The quantitative analysis of defects scored in RNAi-treated and control cells (Table 1) is based on the following numbers: at least 100 prometaphases and metaphases for MS, 70 metaphases for CM, 300 telophases for LIB and CB, 5500 for MS. Three independent experiments were performed.

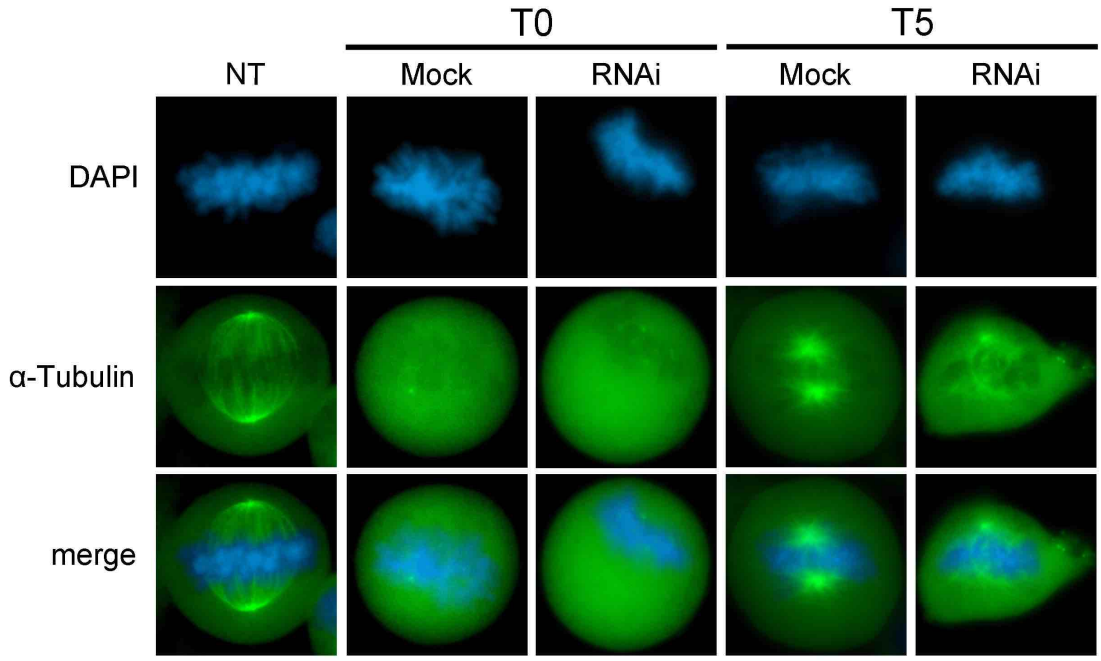

Figure 4. Abnormal microtubules re-polymerization in SRCAP depleted cells.

In control Hela cells microtubule re-polymerization after 5 minutes of rewarming give rise to properly shaped asters, while in SRCAP depleted HeLa cells (RNAi), aster reformation is clearly aberrant with α-Tubulin fluorescence marking only one pole spot, together with sparse and disorganized fibers. The results are based on a total of three experiments; at least 300 cells were scored from both RNAi-treated and control cells; DAPI (blue) and α-Tubulin (green).

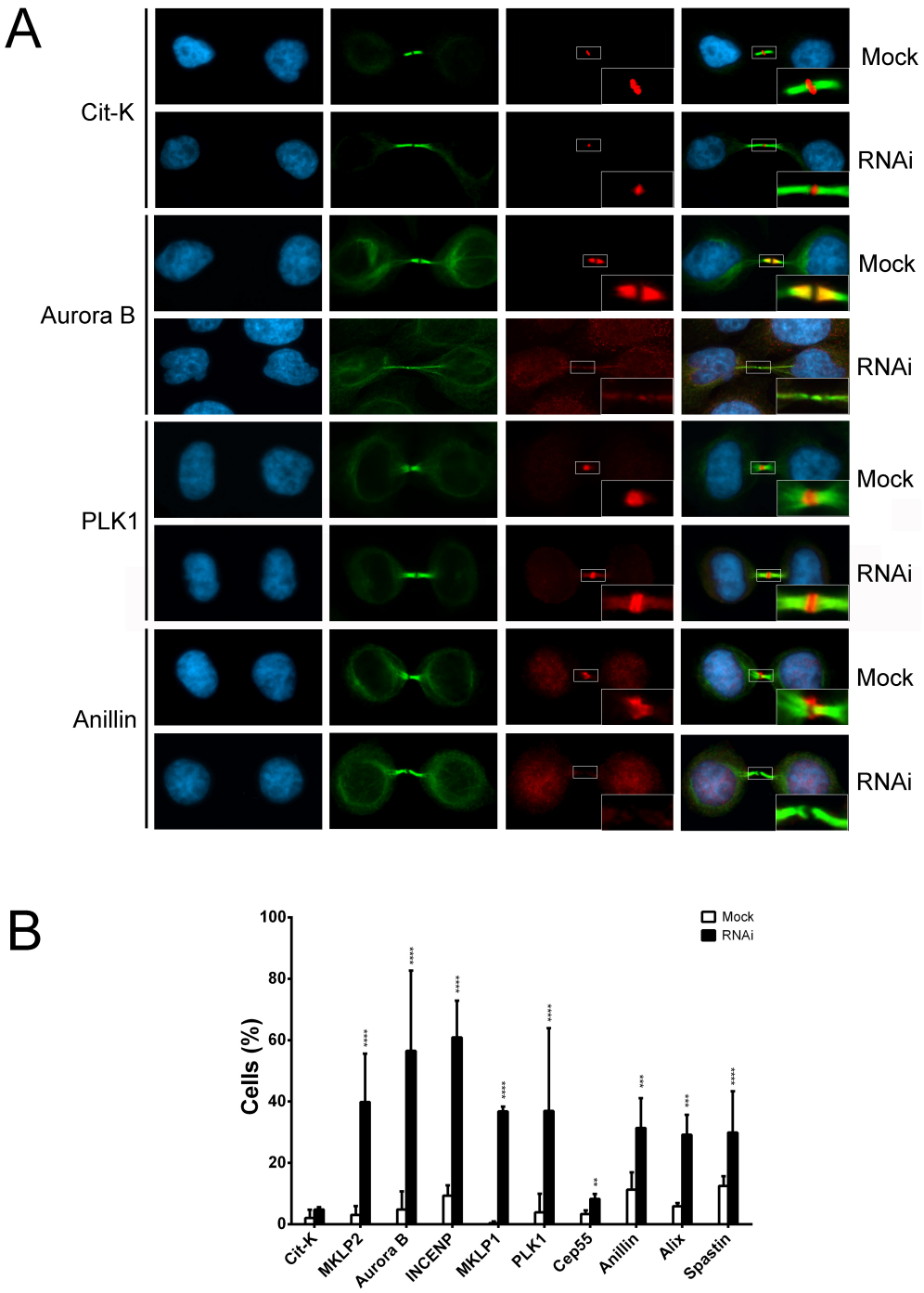

Figure 5. SRCAP depletion affects the midbody localization of cytokinesis regulators.

A) Examples of cytokinesis regulators recruitment at midbody in mock and SRCAP depleted cells (RNAi). B) Histograms showing the quantitative analysis of mis-localizations. Three independent experiments were performed and at least 300 telophases were scored in both RNAi-treated and control cells.

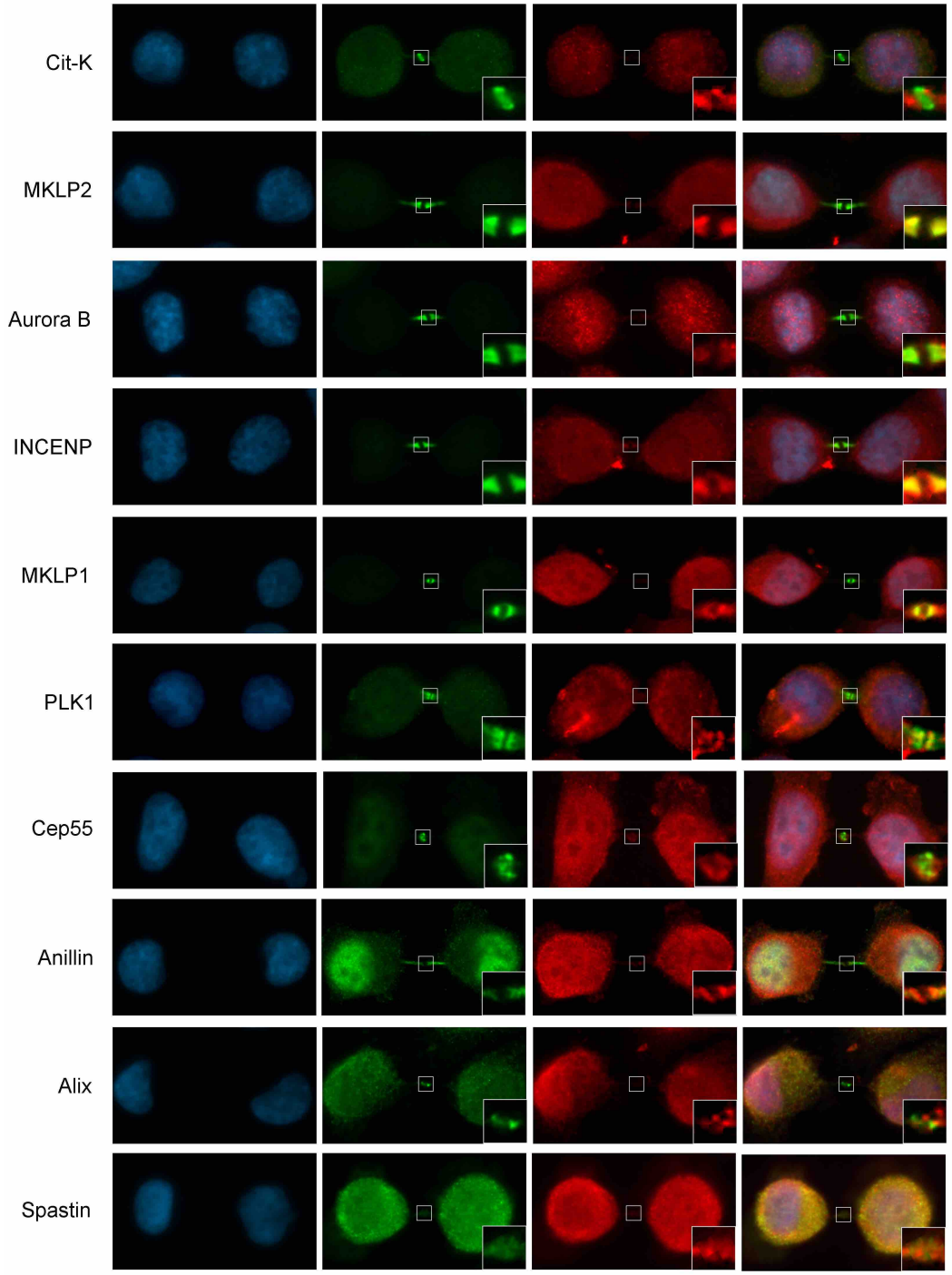

Figure 6. SRCAP colocalizes with cytokinesis regulators at midbody.

The SRCAP signal shows a good overlapping at midbody with those of MKLP1, MKLP2, Aurora B, INCENP, CEP55, Anillin, Alix, and Spastin. Only partial co-localization was seen with CIT-K.

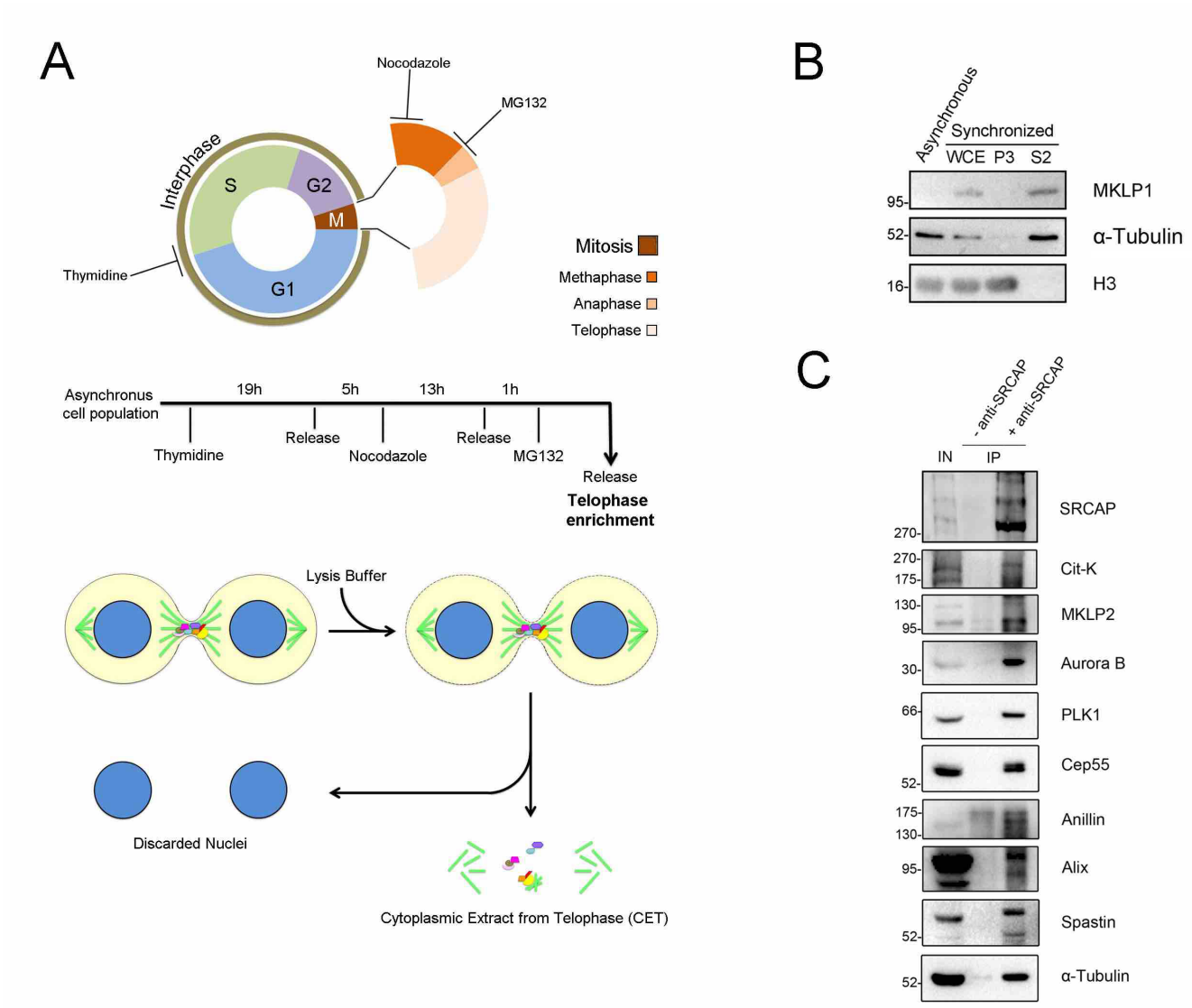

Figure 7. SRCAP interacts with cytokinesis regulators in Co-IP assays.

A) Telophase synchronization in HeLa cells. The scheme summarizes the protocol used for telophase synchronization and subcellular fractionation assay (see Methods). B) Chromatin fractionation of HeLa cells synchronized in telophase. WCE: whole cell extract. P3: nuclear fraction. S2: cytoplasmatic fraction. MKLP1 is expressed in late stages of mitosis (telophase synchronization control). C) Co-immunoprecipitation of protein extracts from telophase synchronized cells (S2 fraction). Three independent IP experiments were performed.

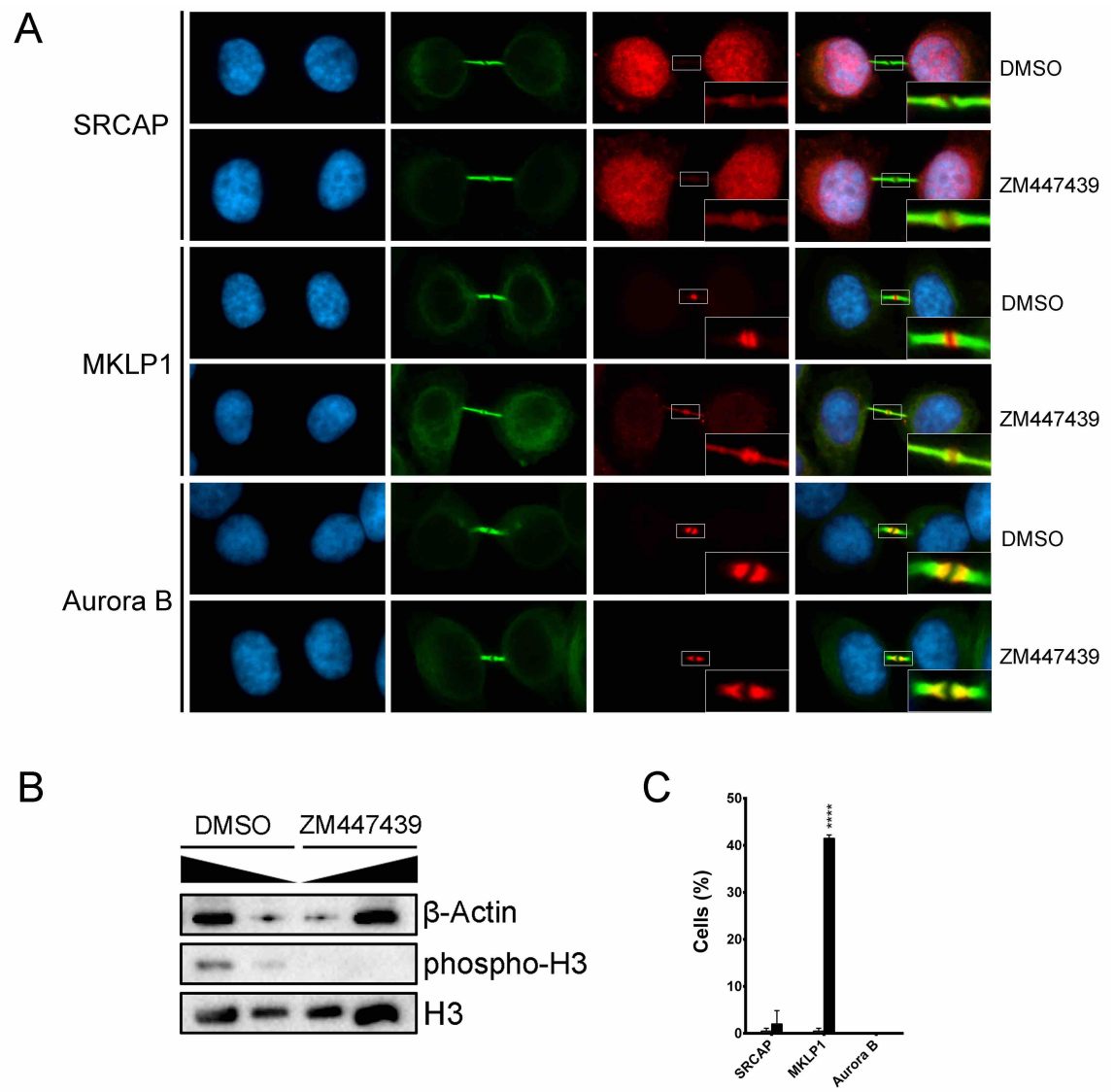

Figure 8. Inhibition of Aurora B kinase activity with ZM447439 does not affect the localization of SRCAP to the midbody.

A) Anti-SRCAP immunostaining of ZM447439-treated HeLa cells versus DMSO-treated control HeLa cells (upper panel); anti-MKLP1 immunostaining positive control (middle panel); anti-Aurora B immunostaining negative control (lower panel). B) To test the efficacy of ZM447439 inhibition, phosphorylation of the histone H3, a target of Aurora B kinase, during mitosis was evaluated. C) Graph showing mis-localization of MKLP1 to the midbody in ZM447439-treated HeLa cells; no effect on SRCAP and Aurora B localizations was found. At least of 300 telophases were scored in three independent experiments for both treated cells and control HeLa cells.

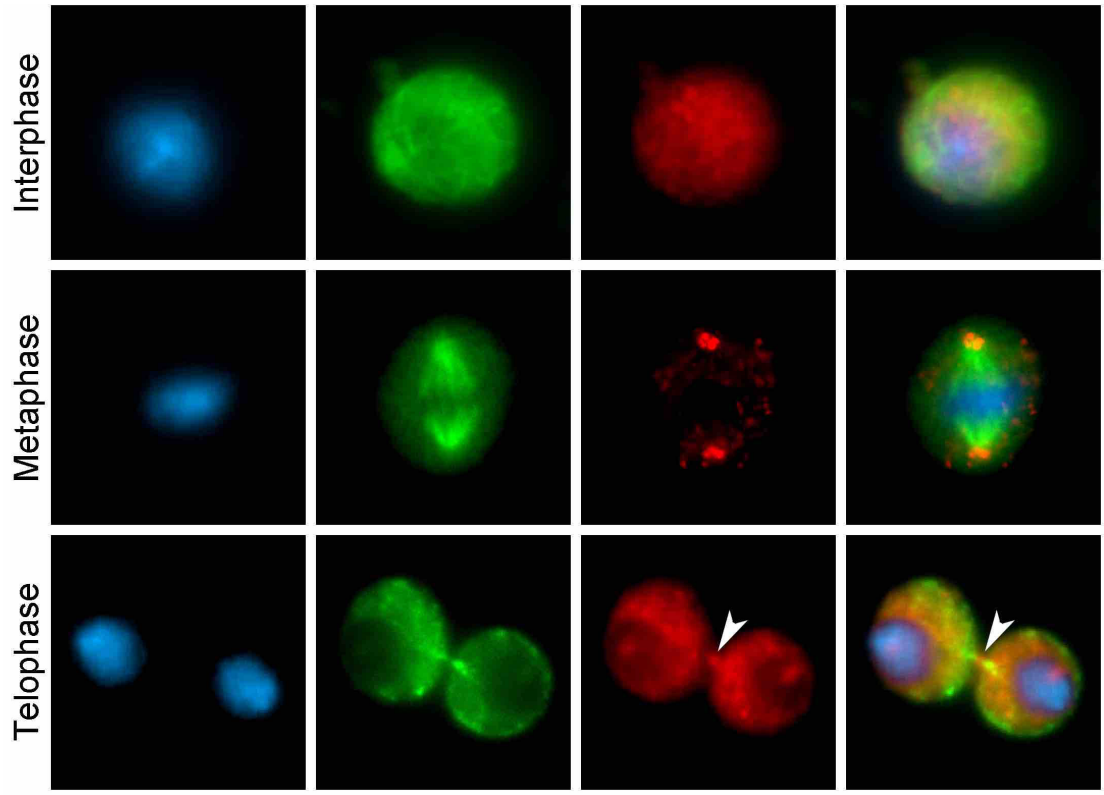

Figure 9. DOM-A localizes to the centrosomes and midbody in S2 cells.

S2 cells were stained with DAPI (blue), anti-α-Tubulin (green) and anti-DOM-A (red). In addition to interphase nuclei, the anti-DOM-A staining was found on centrosomes (metaphase) and midbody (telophase) pointed by an arrow.

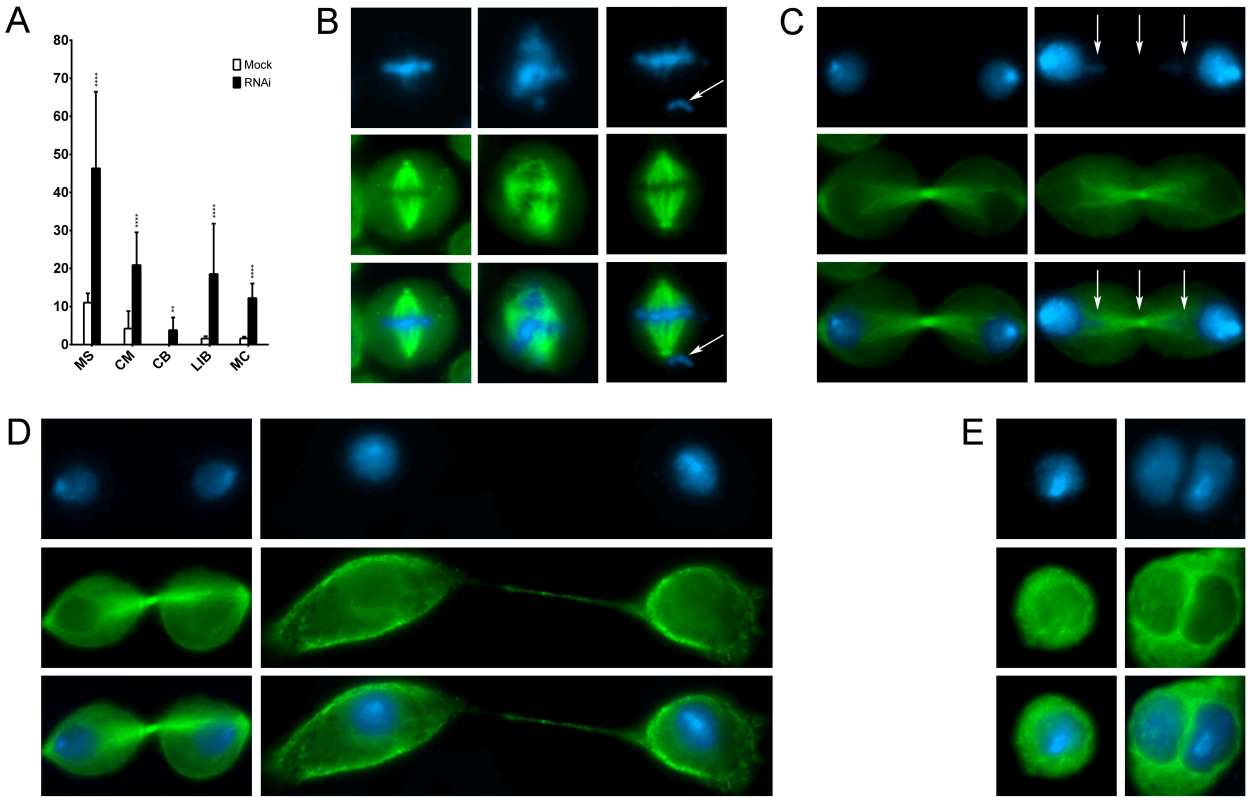

Figure 10. DOM-A depletion in S2 cells affects mitosis and cytokinesis.

Examples of mitotic defects found in DOM-A depleted S2 cells and their and quantification. DAPI staining is shown in blue, α-Tubulin in green. RNAi knock-down experiments were performed by transfecting S2 cells with specific siRNAs (see Methods). Five classes of defects were considered: A) Quantitative analysis defects; B) multipolar spindle (MS) and chromosome misalignments (CM); C) chromatin bridges (CB); D) multinucleated cells (MC); E) long intercellular bridges (LIB). The quantitative analysis of defects scored in RNAi-treated and control cells (see Table 1) is based on about 300 metaphases, telophases or interphases, scored in at least three independent experiments.

TABLES

Table 1. Cell division defects found in SRCAP depleted HeLa cells

		SRCAP	
		Mock	RNAi
Metaphase	MS	4,38 ± 2,58[a]	6,21 ± 4,43
	CM	15,31 ± 14,45	57,36 ± 16,78*
Telophase	CB	6,00 ± 1,97	8,45 ±4,96*
	LIB	1,87 ± 0,67	28,69 ± 9,21*
	MC	2,16 ± 0,49	14,21 ± 3,74*

MS = Multipolar Spindles; CM = Cromosome Misalignments; CB= chromatin bridges

LIB = Long Intercellunar Bridges; MC = Multinucleated Cells;

[a]All results are expressed as mean ± SD values from three independent replicate experiments

Table 2. Cytokinesis regulators misslocalizations in SRCAP depleted HeLa cells

	Mock	SRCAP
CIT-K	2,04 ± 2,65[a]	4,80 ± 0,75
MKLP2	3,03 ± 2,90	39,84 ± 15,78*
Aurora B	4,81 ± 5,90	56,5 ± 26,16*
INCENP	9,28 ± 3,40	60,90 ± 11,93*
MKLP1	0,37 ± 0,57	36,78 ± 1,52*
PLK1	3,86 ± 6,06	36,91 ± 27*
Cep55	3,33 ± 1,15	8,27 ± 1,61*
Anillin	11,26 ± 5,66	31,38 ± 9,67*
Alix	5,83 ± 1,06	29,15 ± 6,48*
Spastin	12,4 ± 3,18	29,90 ± 13,43*

[a]All results are expressed as mean ± SD values from three independent replicate experiments.

*=P < 0.05, compared with the Mock group

Table 3. Treatment with ZM447439 inhibitor

	DMSO	ZM447439
SRCAP	0,50 ± 0,58[a]	2,00 ± 2,83
MKLP1	0,50 ± 0,58	41,50 ± 0,71*
Aurora B	0	0

[a]All results are expressed as mean ± SD values from at least three independent replicated experiments.
*=P < 0.05, compared with the Mock group.

Table 4. Quantitative analysis of defects found in DOM-A depleted S2 cells

		DomA	
		Mock	RNAi
Metaphase	MS	11,01 ± 2,48[a]	46,33 ± 20,14*
	CM	4,22 ± 4,58	20,9 ± 8,6*
Telophase	CB	0	3,81 ± 0,50*
	LIB	1,57 ± 0,6	18,54 ± 13*
	MC	1,16 ± 0,5	12,21 ± 3,84*

[a]All results are expressed as mean ± SD values from three independent replicated experiments.

*=P < 0.05, compared with the Mock group.

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