

# Mitotic Functions for SNAP45, a Subunit of the Small Nuclear RNA-activating Protein Complex SNAP<sub>c</sub><sup>\*[S]</sup>

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The small nuclear RNA-activating protein complex SNAP<sub>c</sub> is required for transcription of small nuclear RNA genes and binds to a proximal sequence element in their promoters. SNAP<sub>c</sub> contains five types of subunits stably associated with each other. Here we show that one of these polypeptides, SNAP45, also known as PTF δ, localizes to centrosomes during parts of mitosis, as well as to the spindle midzone during anaphase and the mid-body during telophase. Consistent with localization to these mitotic structures, both down- and up-regulation of SNAP45 lead to a G<sub>2</sub>/M arrest with cells displaying abnormal mitotic structures. In contrast, down-regulation of SNAP190, another SNAP<sub>c</sub> subunit, leads to an accumulation of cells with a G<sub>0</sub>/G<sub>1</sub> DNA content. These results are consistent with the proposal that SNAP45 plays two roles in the cell, one as a subunit of the transcription factor SNAP<sub>c</sub> and another as a factor required for proper mitotic progression.

Many biological processes are combinatorial, using the principle of mixing limited numbers of individual elements to give rise to nearly unlimited numbers of combinations with different functional attributes. A classical example occurs in promoters, where different arrangements of sequence elements result in the recruitment of different combinations of transcription factors that can provide the complex regulation needed for processes such as differentiation and development. Another example is in the repeated use of various polypeptides in different protein complexes. In some cases, such as the TBP-associated factors (TAFs) present in both the transcription factor IID (TFIID) and Spt-Ada-Gen5 acetyltransferase (SAGA) complexes (1, 2), the resulting complexes are involved in the same general process, in this example transcription. In other cases, however, the same proteins can exert their effect in completely different processes; for example, glyceraldehyde-3-phosphate dehydrogenase functions as a glycolytic enzyme in the cytoplasm as well as a member of a

nuclear co-activator complex involved in cell cycle-regulated transcription from the *H2B* promoter (3). This last theme is becoming more and more common as we learn more about the players in various cellular processes.

The snRNA-activating protein complex SNAP<sub>c</sub> is a multi-subunit complex containing five types of subunits, SNAP190, SNAP50, SNAP45, SNAP43, and SNAP19, that is required for RNA polymerase II and III transcription of the human snRNA<sup>2</sup> genes (for a review see Ref. 4). The arrangement of the subunits within the complex has been deduced from protein-protein interaction studies and reconstitution of partial complexes *in vitro*. SNAP190 forms the backbone of the complex and binds three of the four remaining subunits through two main regions as follows: a region within the N-terminal third binds SNAP19 and SNAP43, whereas a region close to the C terminus of the protein binds SNAP45. SNAP50 joins the complex through contacts with SNAP43 (5–8). A subcomplex of SNAP<sub>c</sub>, referred to as mini-SNAP<sub>c</sub>, missing the C-terminal two-thirds of SNAP190 and associated SNAP45, is still capable of directing *in vitro* transcription of RNA polymerase II and III snRNA genes, albeit with lower efficiency than complete SNAP<sub>c</sub> (7).

We find that SNAP45, but not the backbone SNAP<sub>c</sub> subunit SNAP190, localizes to the centrosomes during specific stages of mitosis as well as to the spindle midzone during anaphase and the mid-body during telophase. Both down-regulation and overexpression of SNAP45 result in abnormalities in mitotic progression, strongly suggesting that besides its role within the transcription factor SNAP<sub>c</sub>, SNAP45 performs a second essential function during cell division. Thus, SNAP45 is an example of a protein with two very different functions, the first as a subunit of the transcription factor SNAP<sub>c</sub> (9) and the second as a protein involved in mitosis.

## EXPERIMENTAL PROCEDURES

*Cell Culture, Stable Cell Lines, and SNAP45 Expression in E. coli*—HeLa cells were grown in Dulbecco's modified Eagle's medium containing low glucose (Invitrogen) supplemented with penicillin/streptomycin and 10% fetal bovine serum (Hyclone, Logan, UT). For transfection and cell cycle experiments, HeLa cells were grown without any drug in the medium. Cells were transfected with a pBabe derivative (pBabe(I)-HA-SNAP45) expressing SNAP45 carrying a hemagglutinin-de-

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[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S6.

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<sup>2</sup> The abbreviations used are: snRNA, small nuclear RNA; siRNA, silencing RNA; BSA, bovine serum albumin; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; BrdUrd, bromodeoxyuridine; DAPI, 4',6-diamidino-2-phenylindole; Orc, origin recognition complex.

## Mitotic Functions of the SNAP<sub>c</sub> Subunit SNAP45

rived tag (HA tag) at its N terminus, with the FuGENE 6 reagent (Roche Applied Science) according to the manufacturer's protocol. Forty eight hours after transfection, puromycin was added to the medium at a concentration of 2  $\mu\text{g}/\text{ml}$ . Clonal cell lines were derived by serial dilution and individual clones screened for expression of HA-SNAP45 by immunoblot.

For bacterial expression, a pSBet derivative (pSBet-FLAG-SNAP45-His) expressing SNAP45 with an N-terminal FLAG and a C-terminal His tag was transformed into BL21 LysS host cells and expressed by induction with isopropyl 1-thio- $\beta$ -D-galactopyranoside at room temperature. Recombinant SNAP45 was purified over a Ni<sup>2+</sup> affinity column (Qiagen), and the eluted fractions were dialyzed against a buffer containing 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5% glycerol, and 1 mM phenylmethylsulfonyl fluoride.

**RNA Interference**—The siRNAs S3 (GGC UGG UCC CUC CAC UGA A) and S4 (CGA GCA CAG CGA ACU GAA A) directed against SNAP45, or SNAP190-2 (GCA GAA UUG UCU ACU AUA U) and SNAP190-3 (CGU GGA GAU CUC AGA AUC A) directed against SNAP190, or a control siRNA (Qiagen, catalog number 1027281) were purchased from Qiagen. The siRNAs were delivered into the cells at a final concentration of 16 nM with the HiPerFect transfection reagent (Qiagen). The cells were transfected twice at an interval of 24 h and then used for immunoblotting, FACS, and indirect immunofluorescence analyses.

**Indirect Immunofluorescence Analyses**—Cells were rinsed in PBS (pH 7.4) and then fixed in either 2.0% paraformaldehyde/PBS (pH 7.4) for 15 min or in cold methanol (−20 °C) for 5 min as indicated in the figure legends. In the case of paraformaldehyde fixation, the cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature. The cells were rinsed three times with PBS, incubated with the primary antibody for 1 h at room temperature, and then with the secondary antibodies for 1 h at room temperature (Invitrogen, Alexa Fluor-594 goat anti-rabbit IgG, Alexa Fluor-488 goat anti-mouse IgG). The cells were counterstained with DAPI (Pierce) and mounted with mounting medium (Vector Laboratories). The cells were then examined with a fluorescence microscope (Axioplan 2i; Carl Zeiss MicroImaging Inc.), and digital images were collected with the Open Lab Software (Improvision).

The primary antibodies used in this study were the anti-SNAP45 rabbit polyclonal antibody (SZ2809), raised against the SNAP45 peptide AEGDGAGSKAPEETP-CONH<sub>2</sub> with an additional cysteine at the N terminus and affinity-purified against recombinant SNAP45 protein, as well as anti- $\alpha$ -tubulin (clone B-5-1-2, Sigma), anti- $\gamma$ -tubulin (clone GTU-88, Sigma), anti-aurora B (Sigma), anti-HA (HA.11, Covance), anti-cyclin A (clone E23, Invitrogen), anti-cyclin B (BD Biosciences), anti-cyclin D1 (SC-753, Santa Cruz Biotechnology), anti-cyclin E (clone HE12, Invitrogen), anti-H3-Ser-10 (clone 3H10, Upstate), and anti-polo-like kinase (mouse anti-plk1, Invitrogen) antibodies. We also used an anti-hCAP-G antibody, a kind gift of T. Hirano (Cold Spring Harbor Laboratory), and an anti-caspase 9 antibody, a kind gift of Y. Lazebnik (Cold Spring Harbor Laboratory).

**Flow Cytometry**—Cells were fixed with cold methanol, stained with propidium iodide (2  $\mu\text{g}/\text{ml}$ ), and analyzed with a LSRII cell analyzer (BD Biosciences). For cell sorting, exponen-

tially growing HeLa S3 cells were stained with Hoechst 3342-A at 10  $\mu\text{g}/\text{ml}$  for 30 min, washed, resuspended in fresh medium, and sorted with a FACSVantage SE cell sorter (BD Biosciences). The separated cells were pelleted and frozen for further analysis.

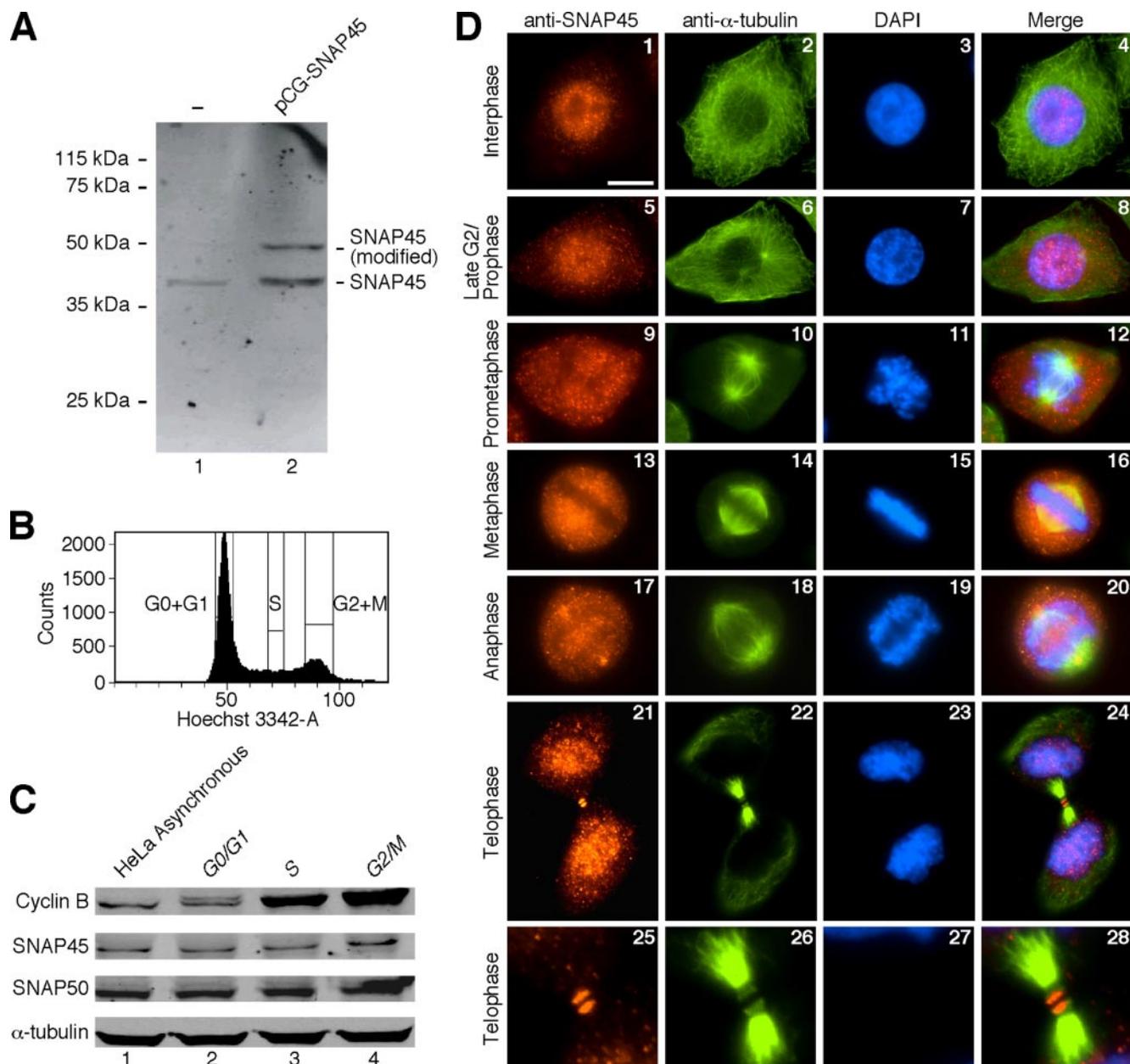
**Metaphase Spreads**—Mitotic cells were collected by shake off and centrifuged at 1000 rpm for 5 min. The cell pellet was washed with PBS, resuspended in 0.075 M KCl, and incubated for 30 min at 37 °C. The cells were then centrifuged at 1000 rpm for 5 min, and the cell pellet was resuspended in a minimal volume of 0.075 M KCl and cytospun onto clean polylysine-coated coverslips. The cells were fixed with 2.0% paraformaldehyde for 15 min at room temperature and prepared for indirect immunofluorescence microscopy as described above.

**BrdUrd Labeling**—BrdUrd labeling was performed as described (10). Briefly, cells were incubated with 10 mM BrdUrd (Pharmingen) for 10 min at 37 °C, washed with PBS, and fixed with 2% paraformaldehyde for 15 min at room temperature. Cells were then washed, permeabilized with 0.5% Triton X-100 in PBS for 5 min, incubated with the anti-SNAP45 antibody SZ2809, and followed by the secondary antibody Alexa Fluor-594 goat anti-rabbit IgG. Cells were washed three times for 10 min with PBS and fixed with 2% paraformaldehyde for 15 min at room temperature. The DNA was then denatured with 4 N HCl for 30 min at room temperature, and the cells were washed with PBS and incubated with an anti-BrdUrd antibody conjugated with fluorescein isothiocyanate (Roche Applied Science) for 1 h at room temperature. The cells were then stained with DAPI (Pierce) and mounted.

**In Vitro Phosphorylation Assay**—For the *in vitro* phosphorylation assays, ~5–10 pmol of SNAP45 and, as a positive control, Orc2 (11) were incubated in 40  $\mu\text{l}$  of kinase buffer (50 mM HEPES (pH 7.0), 10 mM MgCl<sub>2</sub>, 4 mM MnCl<sub>2</sub>, 1 mM dithiothreitol, 0.1 mg/ml BSA where indicated, and 2  $\mu\text{Ci}$  of [ $\gamma$ -<sup>32</sup>P]ATP) for 30 min at 30 °C in the presence of the indicated amounts of either purified cyclin A/Cdk2, cyclin E/Cdk2, or cyclin B/Cdk1 (Upstate). The reactions were stopped with Laemmli buffer and subjected to SDS-PAGE, and the gels were autoradiographed.

## RESULTS

**Centrosome and Mid-body Staining with an Anti-SNAP45 Antibody**—In routine tests of anti-SNAP45 antibodies, we noticed surprising staining patterns in immunofluorescence experiments and therefore decided to examine them more closely. The quality of the affinity-purified antibody can be assessed from the Western blot of whole HeLa cell lysates shown in Fig. 1A. The antibody recognized one main band (Fig. 1A, lane 1) co-migrating with the darker band observed after transfection of the HeLa cells with a construct (pCG-SNAP45, lane 2) expressing untagged SNAP45. (The second, slower migrating band observed in transfected cells corresponds to ubiquitylated SNAP45 as determined by co-transfection of the cells with a vector expressing His-tagged ubiquitin followed by immunostaining of the nickel agarose-binding proteins with an anti-SNAP45 antibody; data not shown.) As described below (see Fig. 3A), this signal was specifically diminished after treatment of the cells with two different siRNAs directed against



**FIGURE 1. Localization of SNAP45 during the cell cycle.** *A*, reactivity of the anti-SNAP45 antibody. Whole cell extract from mock-transfected HeLa cells (*lane 1*) or from HeLa cells transfected with pCG-SNAP45, a vector expressing untagged SNAP45 from a strong (cytomegalovirus) promoter (*lane 2*), was fractionated on an SDS-polyacrylamide gel and subjected to immunoblotting with a polyclonal rabbit anti-peptide antibody directed against SNAP45 (rabbit SZ2809). *B*, asynchronous HeLa S3 cells were subjected to cell sorting, and gated samples were collected for immunoblot analysis. *C*, immunoblot analysis of SNAP45 in cell cycle-staged cells. Samples from asynchronous cells or from cells with G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M DNA contents were subjected to immunoblot analysis with antibodies directed against cyclin B (BD Biosciences), SNAP45 (SZ2809), SNAP50 (CS303), and  $\alpha$ -tubulin (clone B-5-1-2, Sigma). *D*, HeLa cells were fixed with 2% paraformaldehyde and stained for indirect immunofluorescence with the anti-SNAP45 (SZ2809) (red) and anti- $\alpha$ -tubulin (clone B-5-1-2, Sigma) (green) antibodies. DNA was stained with DAPI (blue). Scale bar, 10  $\mu$ m. Panels 25–28 show a blow-up of the midbody region in panels 21–24.

SNAP45 mRNA, strongly suggesting that it indeed corresponds to SNAP45. A Western blot analysis of lysates from asynchronous cells and cells in G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M phases sorted according to DNA content (Fig. 1*B*) revealed no change in an  $\alpha$ -tubulin control signal, as expected (Fig. 1*C*). The cyclin B signal was greatly increased in M phase cells, as expected, but also showed an unexpected increase in S phase cells, suggesting a contamination of this cell population by G<sub>2</sub>/M phase cells. At any rate, however, there was no significant difference in SNAP45 amounts or migration during the cell cycle (Fig. 1*C*; note that the apparent slower migration of SNAP45 in lanes 3

and 4 reflects warping of the gel), and the same was true for SNAP50.

Nonsynchronized HeLa cells were then stained with the anti-SNAP45 antibody (Fig. 1*D*, red), an anti- $\alpha$  tubulin antibody (green) to visualize the microtubule network, and DAPI (blue) to visualize DNA. Fig. 1*D* shows the results obtained after paraformaldehyde fixation of the cells. In interphase cells, the staining was mainly nuclear, consistent with the role of SNAP45 in transcription, although some weak cytoplasmic staining, which may correspond to background, was visible (Fig. 1*D*, panels 1–4). During late G<sub>2</sub>/early prophase, the

## Mitotic Functions of the SNAP<sub>c</sub> Subunit SNAP45

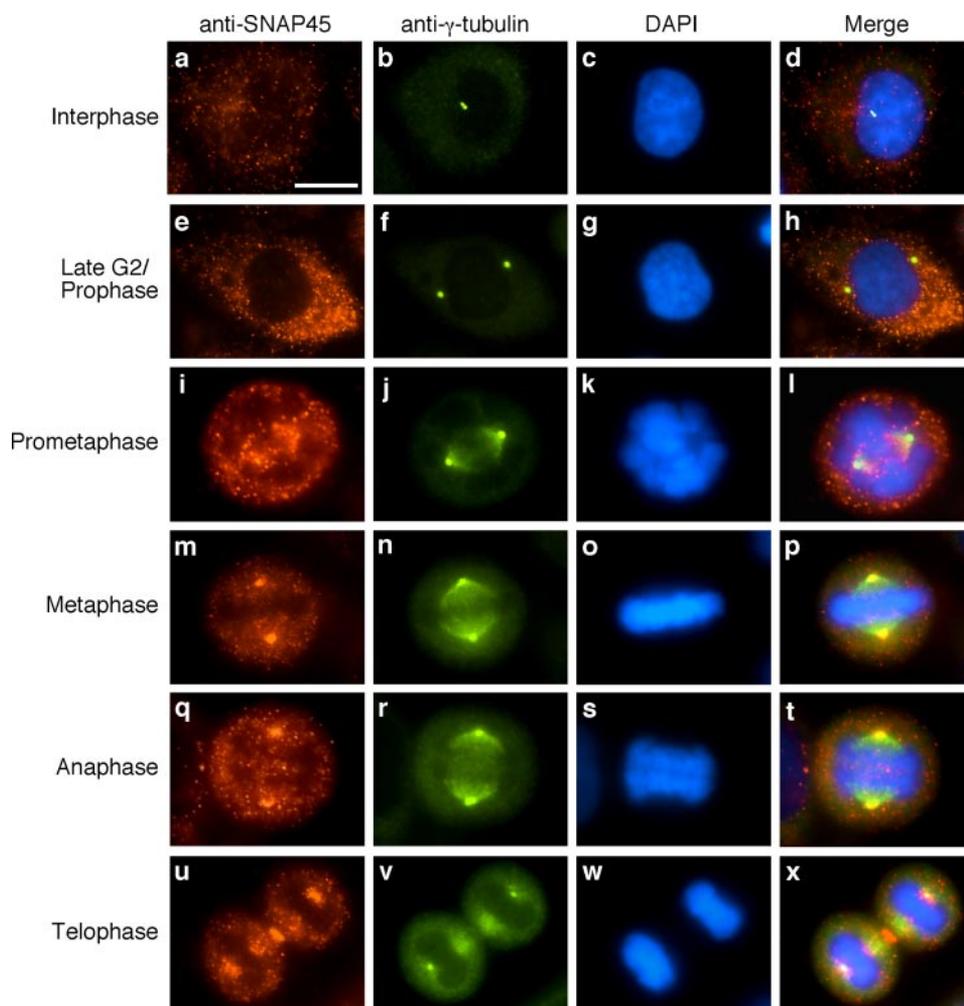


FIGURE 2. **SNAP45 localization to centrosomes during mitosis.** HeLa cells were fixed with ice-cold methanol and stained for indirect immunofluorescence with anti-SNAP45 (SZ2809) (red) and anti- $\gamma$ -tubulin (clone GTU-88, Sigma) (green) antibodies. DNA was stained with DAPI (blue). Scale bar, 10  $\mu$ m.

SNAP45 staining was still largely within regions of DAPI staining (Fig. 1D, panels 5–8), but starting in prometaphase and up to anaphase, it was largely nonoverlapping with the DAPI staining of DNA (panels 9–20). In telophase, the SNAP45 staining started to concentrate again in the two newly formed nuclei (Fig. 1D, panels 21–24). Thus, SNAP45 is mostly nuclear in interphase cells but then seems to separate from the DNA during cell division.

During anaphase, we observed a concentration of SNAP45 staining in the spindle pole regions, as well as in the spindle midzone (Fig. 1D, panels 17–20), which was then concentrated in the mid-body at telophase (Fig. 1D, panels 21–28). To visualize these structures better, we used methanol fixation. As shown in supplemental Fig. 1A, methanol fixation resulted in a loss of much of the DNA-overlapping SNAP45 signal in late G<sub>2</sub>/early prophase and telophase cells (panels a–d and q–t), but this fixation method allowed better visualization of the spindle pole region. Indeed, a concentration of SNAP45 staining could be seen in the spindle pole region in metaphase cells already (supplemental Fig. 1A panels i–l), which persisted during anaphase and telophase (panels m–t). Moreover, the staining in the spindle midzone region was again apparent in anaphase

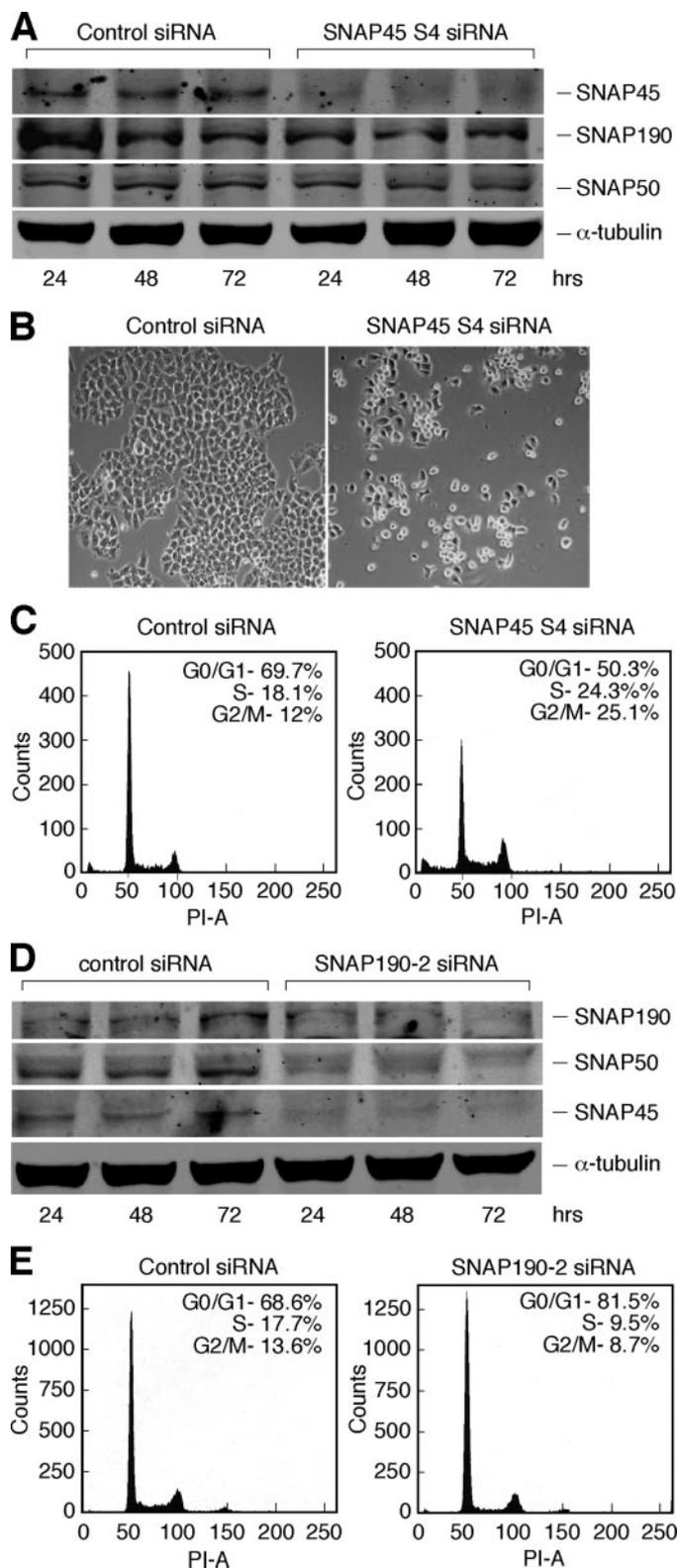
(supplemental Fig. 1A panels m–p), as well as staining of the mid-body in telophase (panels q–t).

We stained two other cell lines, the human embryonic kidney HEK-293 cell line and the human osteosarcoma U2OS cell line, with the anti-SNAP45 antibody. Both the HEK-293 (supplemental Fig. S1B) and U2OS (supplemental Fig. S1C) cells displayed very similar staining patterns after formaldehyde fixation as those observed above with HeLa cells (see Fig. 1D), indicating that this SNAP45 staining pattern is not unique to HeLa cells.

To determine whether the spindle pole regions stained with the anti-SNAP45 antibody corresponded to centrosomes, we stained methanol-fixed cells with both the anti-SNAP45 antibody (Fig. 2, red) and an anti- $\gamma$ -tubulin antibody (green), which marks the centrosomes. As shown in Fig. 2, the anti- $\gamma$ -tubulin antibody marked the centrosomal complex in interphase cells (panel b) and the two separated centrosomes during mitosis (panels f, j, n, r, and v). Starting in metaphase and continuing through anaphase and telophase, there was SNAP45 staining overlapping the  $\gamma$ -tubulin staining, suggesting that some SNAP45 localizes to the centrosomes in metaphase and stays associated

with this structure until telophase (Fig. 2, panels m–x). Thus, the indirect immunofluorescence images reveal a staining pattern that is very unexpected for a transcription factor subunit. We attempted to observe this pattern in living cells with SNAP45-GFP and GFP-SNAP45 fusion proteins, but unfortunately both fusion proteins aberrantly localized to the cytoplasm. Nevertheless, that the staining pattern observed here indeed reflects localization of SNAP45 rather than that of a cross-reacting protein is strongly supported by the results below (see Fig. 4), in which down-regulation of SNAP45 by RNA interference suppresses the staining patterns observed here. Interestingly, none of our antibodies against other SNAP<sub>c</sub> subunits gave staining patterns similar to those observed with the anti-SNAP45 antibody (data not shown), consistent with the idea that SNAP45 may play a role during mitosis that is separate from its role as a subunit of SNAP<sub>c</sub>.

**Down-regulation of Cellular SNAP45 Results in Defects in Mitotic Progression**—The transient association of SNAP45 with the centrosomes during part of mitosis and with the mid-body during telophase prompted us to determine whether the protein might play a role during cell division. HeLa cells were transfected twice at a 24-h interval with two different siRNAs



**FIGURE 3. Down-regulation of SNAP45 results in accumulation of cells with S and G<sub>2</sub>/M DNA contents.** *A*, down-regulation of SNAP45 by siRNAs. HeLa cells were transfected two times in a 24-h interval with a control siRNA and two different siRNAs against SNAP45 RNA. Samples were collected 24, 48, and 72 h after the second transfection and analyzed by immunoblot with anti-SNAP45, anti-SNAP190 (CS696), anti-SNAP50, and anti- $\alpha$ -tubulin (as a loading control) antibodies as in Fig. 1C. The results with siRNA S4 directed against SNAP45 are shown. *B*, phase contrast light microscopy pictures showing an accumulation of rounded mitotic cells after depletion of SNAP45 with the SNAP45 S4 siRNA. *C*, FACS analysis of control and SNAP45 S4

directed against SNAP45. As shown in Fig. 3A by Western blot, the siRNA S4 efficiently down-regulated SNAP45 as early as 24 h after the second transfection, as did the second siRNA directed against SNAP45 (S3; data not shown). In contrast, the levels of the SNAP<sub>c</sub> subunits SNAP190 and SNAP50 were only slightly diminished, indicating that SNAP45 is not essential for the stability of other SNAP<sub>c</sub> subunits.

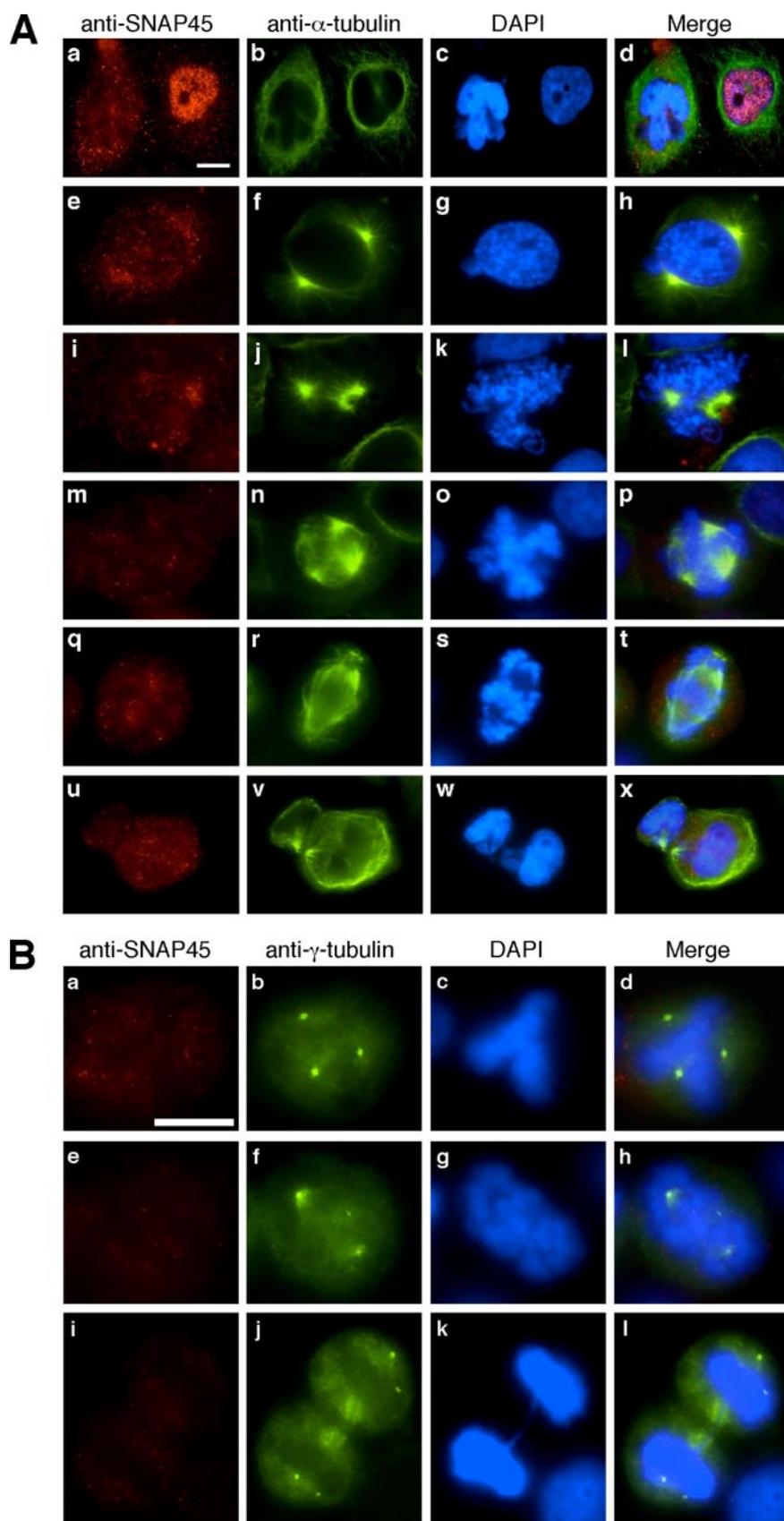
Inspection of cells transfected with a control siRNA *versus* cells transfected with the anti-SNAP45 siRNA S4 by phase contrast microscopy revealed a striking accumulation of rounded cells (Fig. 3B), suggesting mitotic arrest. Indeed, FACS analysis showed an increase of S phase cells from about 18 to 24% and strikingly a more than 2-fold increase in G<sub>2</sub>/M cells from 12 to 25% after anti-SNAP45 siRNA transfection (Fig. 3C). Similar effects were observed with the second anti-SNAP45 siRNA, S3, although the phenotypes were slightly milder, strongly suggesting that they did not result from off-target effects of the siRNAs. A Western blot analysis of SNAP45, cyclins B, A, D1, and E, Aurora B, and polo-like kinase 1 at different times after siRNA transfection, shown in supplemental Fig. S2, revealed, as expected, a dramatic decrease in SNAP45 after siRNA treatment (supplemental Fig. S2, compare lanes 4–6 with lanes 1–3). In addition, there was a slight increase in cyclins B and D1, consistent with an increase in cells with G<sub>2</sub>/M DNA contents, and a decrease in cyclin E, probably reflecting the decrease of G<sub>1</sub> and perhaps early S phase cells. The levels of the other proteins tested remained quite constant.

To characterize the abilities of cells depleted of SNAP45 to progress through S and G<sub>2</sub> phases, we checked DNA synthesis. As shown in supplemental Fig. S3, *panel A*, cells even severely depleted of SNAP45 still incorporated BrdUrd. A survey of 200 cells indicated that 21% of cells transfected with the control siRNA incorporated BrdUrd, for 35% of cells transfected with the siRNA directed against SNAP45. This is consistent with the increase in S phase cells observed by FACS analysis above and suggests that in these cells DNA synthesis is continuing. We also checked for phosphorylation of serine 10 on histone H3, a mitotic marker, and the results are shown in supplemental Fig. S3, *panel B*. Cells depleted of SNAP45 in early prophase (supplemental Fig. S3, *panels i–p*) or prometaphase (Fig. S3, *panels q–t*) stained normally for histone H3 phosphorylated at serine 10 as compared with cells transfected with the control siRNA in the same stages (early prophase, Fig. S3, *panels a–d*, and prometaphase, Fig. S3, *panels e–h*), suggesting normal Aurora B activity.

As mentioned above, the specific staining of centrosome and mid-body structures observed with anti-SNAP45 antibodies was not observed with antibodies directed against other SNAP<sub>c</sub> subunits (data not shown). Therefore, we wondered whether the accumulation of S and G<sub>2</sub>/M phase cells resulted from down-regulation of the entire SNAP<sub>c</sub> or from specific down-regulation of SNAP45, as suggested by the only slightly changed

siRNA-treated cells. *D*, down-regulation of SNAP190 by siRNA. HeLa cells were transfected as in *A* but with control siRNA or siRNA SNAP190-2 directed against SNAP190. Samples were collected 24, 48, and 72 h after the second transfection and analyzed by immunoblot with anti-SNAP45, anti-SNAP190, anti-SNAP50, and anti- $\alpha$ -tubulin (as a loading control) antibodies as in *A*. *E*, FACS analysis of control and SNAP190-2 siRNA-treated cells.

## Mitotic Functions of the SNAP<sub>c</sub> Subunit SNAP45



**FIGURE 4. Down-regulation of SNAP45 leads to mitotic defects.** *A*, HeLa cells transfected with SNAP45 S4 siRNA were fixed with 2% paraformaldehyde and stained for indirect immunofluorescence with anti-SNAP45 (red) and anti- $\alpha$ -tubulin (green) antibodies. DNA was stained with DAPI (blue). Scale bar, 10  $\mu$ m. *B*, HeLa cells transfected with SNAP45 S4 siRNA were fixed with ice-cold methanol and stained for indirect immunofluorescence with anti-SNAP45 (red) and anti- $\gamma$ -tubulin (green) antibodies. DNA was stained with DAPI (blue). Scale bar, 10  $\mu$ m.

levels of SNAP190 and SNAP50 after SNAP45 depletion (see above Fig. 3A). To address this question, we transfected cells with an siRNA directed against SNAP190, the largest SNAP<sub>c</sub> subunit, which forms the backbone of the complex. As shown in Fig. 3D, SNAP190 protein levels were severely decreased, and there was a concomitant decrease in both SNAP50 and SNAP45 protein levels, likely reflecting destabilization of SNAP<sub>c</sub>. FACS analysis of these cells revealed that, unlike down-regulation of SNAP45 only, down-regulation of SNAP190, SNAP50, and SNAP45 resulted in an accumulation of cells with a G<sub>0</sub>/G<sub>1</sub> DNA content from 68.6 to 81.5%, and a concomitant decrease in cells with S and G<sub>2</sub>/M DNA contents (Fig. 3E), a pattern typical for down-regulation or inactivation of transcription factors (see for example Ref. 12). The different outcomes of SNAP45 and SNAP190 down-regulation suggest that the mitotic arrest observed upon down-regulation of SNAP45 is not a transcriptional effect resulting from down-regulation of SNAP<sub>c</sub> but rather an effect specific for down-regulation of the SNAP45 polypeptide.

To confirm this point, we used a cell line with an integrated construct expressing an unstable RNA (whose levels therefore reflect ongoing transcription) from the SNAP<sub>c</sub>-dependent RNA polymerase III U6 snRNA promoter (13). As shown in supplemental Fig. S4, the U6 signal was diminished when the cells were transfected with increasing amounts of siRNAs directed against SNAP45 (Fig. S4, lanes 2–5) or SNAP190 (Fig. S4, lanes 8 and 9) compared with cells transfected with increasing amounts of control siRNAs (Fig. S4, lanes 6 and 7), mock-transfected with increasing amounts of buffer alone (Fig. S4, lanes 10 and 11), or untransfected (Fig. S4, lanes 12 and 13; the two lanes show duplicate samples). However, the signal was either less or equally diminished with siRNAs against SNAP45 as compared with siRNAs against SNAP190 (Fig. S4,

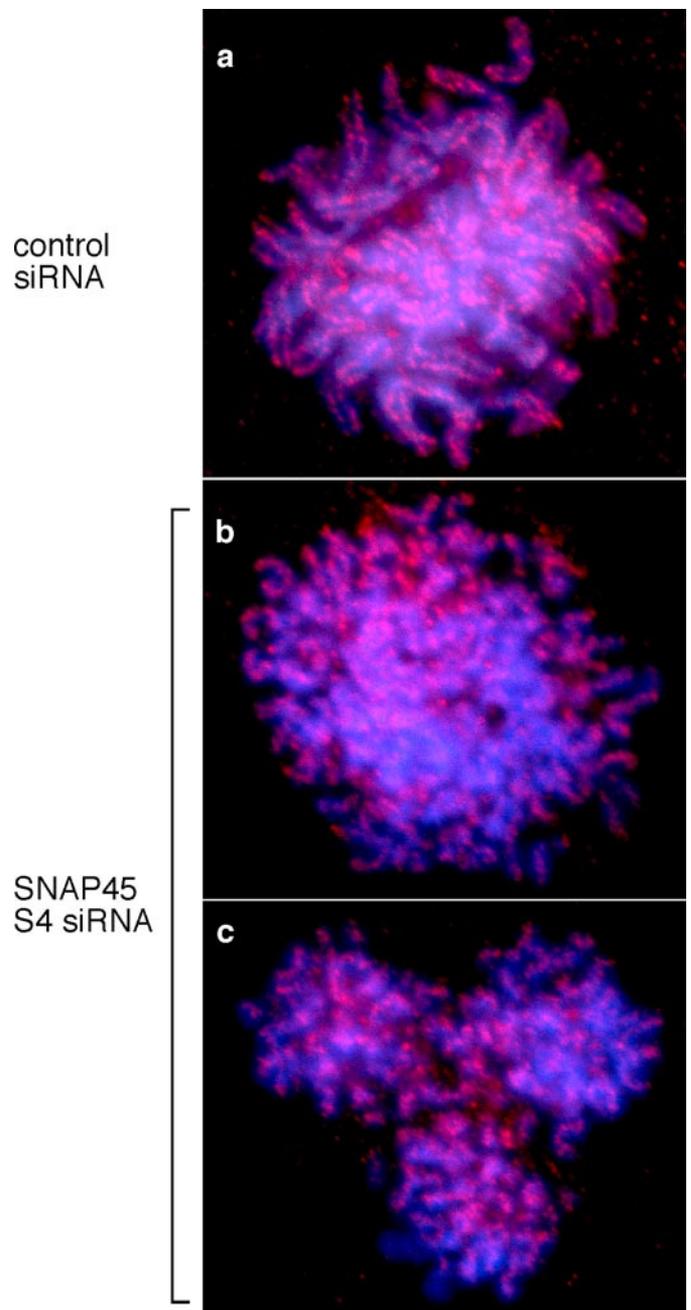
compare lanes 2–5 with lanes 8 and 9), suggesting that down-regulation of SNAP45 did not have a more severe effect on SNAP<sub>c</sub>-dependent transcription, at least by RNA polymerase III, than down-regulation of SNAP190. This further suggests that mitotic arrest results from down-regulation of SNAP45 itself.

Fig. 4A shows examples of cells depleted of SNAP45 as seen by indirect immunofluorescence microscopy after staining with the anti-SNAP45 antibody (*red*), an anti- $\alpha$ -tubulin antibody (*green*), and DAPI (*blue*). The difference in SNAP45 content after anti-SNAP45 siRNA transfection can be seen in Fig. 4A, *panel a*, which shows one cell, presumably transfected by the siRNA, severely depleted of SNAP45 and harboring an irregular-shaped nucleus, and another, presumably untransfected, showing strong SNAP45 staining and a normal nucleus (*panels a–d*). Fig. 4A, *panels e–x*, all show cells severely depleted of SNAP45 at different stages of mitosis. As expected for cells depleted of SNAP45, there is no staining of centrosomes with the anti-SNAP45 antibody (Fig. 4A, see *panels m–p*, showing a cell in metaphase with a tetrapolar spindle, and *panels u–x*, showing a cell in anaphase). Instead, a large number of defects are apparent as follows: irregularly shaped nuclei, micronuclei (Fig. 4A, *panels c, g, and w*), malformed or multipolar spindles (*panels j and n*), improper chromosome localization and alignment during prometaphase and metaphase (*panels k, l and o, p, s, t*), and improper chromosome segregation with lagging chromosomes during anaphase resulting in the abnormal nuclei observed in telophase (*panels w and x*). Such defects were observed in 57% of the cells showing a decrease in SNAP45 and thus presumably transfected with the siRNA directed against SNAP45, compared with 6% of cells transfected with the control siRNA.

Fig. 4B shows SNAP45-depleted, methanol-fixed cells stained with the anti-SNAP45 antibody (*red*), the anti- $\gamma$ -tubulin antibody (*green*) to mark the centrosomes, and DAPI. Multiple or perhaps fragmented centrosomes are clearly visible (Fig. 4B, *panels b, f, and j*). Moreover, DNA trailing is seen at late stage of mitosis (Fig. 4B, *panel k*). Thus, down-regulation of SNAP45 by RNA interference results in multiple mitotic abnormalities.

Mitotic defects can lead to apoptosis (14). Indeed, some SNAP45-depleted cells had condensed nuclei suggestive of apoptosis (see for example Fig. 4A, *panels a–d*, left cell depleted of SNAP45). We therefore checked for activation of procaspase 9 by Western blot, and the results are shown in supplemental Fig. S5. In supplemental Fig. S5, *lane 5* shows a positive control in which HeLa cells were treated with adriamycin, causing the appearance of activated, cleaved caspase 9. Cleaved and activated caspase 9 also appeared at late times in SNAP45-depleted cells but not in cells treated with the control siRNA, suggesting that at least some of the SNAP45-depleted cells eventually undergo apoptosis.

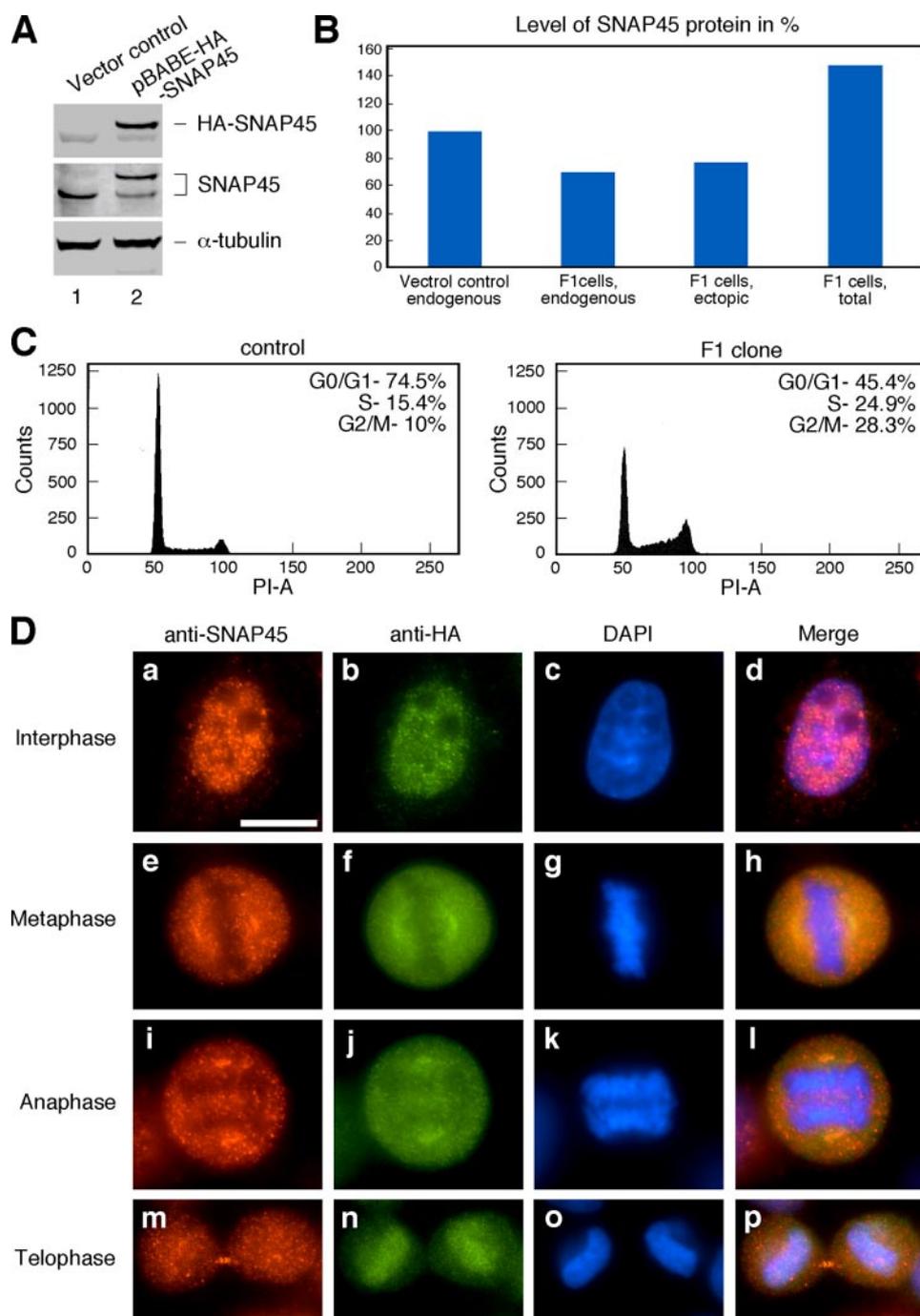
**SNAP45 Depletion Causes Defects in Chromosome Condensation**—The defects observed upon SNAP45 depletion included improper chromosome alignment during metaphase, with some chromosomes failing to localize on the equatorial plate. To examine this possibility more closely, we stained



**FIGURE 5. SNAP45 down-regulation causes chromosome condensation defects.** Metaphase spreads from HeLa cells transfected with control and SNAP45 S4 siRNAs were stained for indirect immunofluorescence with an antibody directed against the non-SMC subunit of the condensin complex h-CAP-G (*red*). DNA was stained with DAPI (*blue*).

metaphase spreads with antibodies directed against the hCAP-G subunit of the condensin complex (*red*) and DAPI, and the results are shown in Fig. 5. In cells treated with the control siRNA, the staining reveals properly paired sister chromatids, as expected (see for example Ref. 15). In contrast, in cells depleted of SNAP45, the chromosomes remained bundled together in the metaphase spread; the CAP-G staining was irregular; DNA regions were devoid of CAP-G staining, and no or very few paired sister chromatids were visible. Thus, SNAP45 depletion causes a major defect in chromosome condensation and sister chromatid pairing at metaphase.

## Mitotic Functions of the SNAP<sub>c</sub> Subunit SNAP45



**FIGURE 6. Overexpression of SNAP45 in HeLa cells.** *A*, immunoblot analysis of SNAP45 expression in early passages of a clonal HeLa cell lines transfected with either empty vector or a vector expressing HA-tagged SNAP45 from a weak (Rous sarcoma virus) promoter (F1 cell line). The blots were probed with monoclonal antibody (HA.11, Covance) directed against the HA tag (*upper panel*), as well as with anti-SNAP45 and anti- $\alpha$ -tubulin antibodies (*middle and lower panels*). *B*, quantitation of the signals in *A* after normalization to the  $\alpha$ -tubulin loading control. *C*, FACS analysis of the control and HA-tagged SNAP45-expressing F1 cell line. *D*, individual F1 cells selected for their normal appearance were fixed with 2% paraformaldehyde and stained for indirect immunofluorescence with anti-SNAP45 (*red*) and anti-HA (HA.11, Covance) (*green*) antibodies. DNA was stained with DAPI (*blue*). Scale bar, 10  $\mu$ m.

**Overexpression of SNAP45 Leads to Multiple Mitotic Defects**—The results above indicate that down-regulation of SNAP45 causes major defects in mitosis. Therefore, we wondered whether overexpression of SNAP45 might also lead to abnormalities. Several clonal cell lines were generated that modestly overexpressed HA-tagged SNAP45. The global level of overexpression

for one of these cell lines, referred to as F1, was about 2-fold, as measured by Western blot (Fig. 6, *A* and *B*). FACS analysis of this cell line, shown in Fig. 6*C*, revealed an accumulation of cells with S and G<sub>2</sub>/M phase DNA contents, from about 15 to 25% for S phase cells and from 10 to 28% for G<sub>2</sub>/M cells. Thus, like down-regulation of SNAP45, overexpression of SNAP45 results in a disruption of the normal cell cycle with accumulation of cells with S and G<sub>2</sub>/M DNA contents.

Because the F1 cell line expresses a tagged version of SNAP45, we then used it to compare the indirect immunofluorescence microscopy signals obtained with the anti-SNAP45 antibody (Fig. 6*D*, *red*) and with an anti-HA antibody (*green*). Fig. 6*D* shows cells selected for their normal or nearly normal aspect. At most cell cycle stages, the anti-HA staining reproduced the patterns observed with the anti-SNAP45 antibody, in particular the nuclear staining in interphase cells (Fig. 6*D*, *panels a–d*), the apparent exclusion of staining from DNA-containing regions during mitosis (*panels e–l*), and the staining of the centrosomes (*panels i–l* showing anaphase cells). Curiously, however, the anti-HA staining pattern of the overexpressed protein differed from that of the endogenous protein in telophase (Fig. 6*D*, *panels m–p*), with the overexpressed protein localizing into the newly forming nuclei much faster than the endogenous protein (Fig. 6*D*, compare *panels m* and *n*, see also Fig. 7*A*, *panels u* and *v*). Moreover, midbody staining was not detected with the anti-HA antibody (compare Fig. 6*D*, *panels m* and *n*, see also Fig. 7*A*, *panels u–x*). Although this could indicate that part of the staining observed with the anti-SNAP45 antibody in telophase results from a cross-reaction with another protein, the disappearance of the signal, including the midbody staining, after transfection with siRNAs directed against SNAP45 (see above, Fig. 4, *panels u–x*) suggests otherwise. Likely possibilities are that the N-terminal HA tag of the overexpressed SNAP45 alters its telophase localization and in particular prevents localization in the mid-body, or that the HA epitope is masked in mid-bodies.

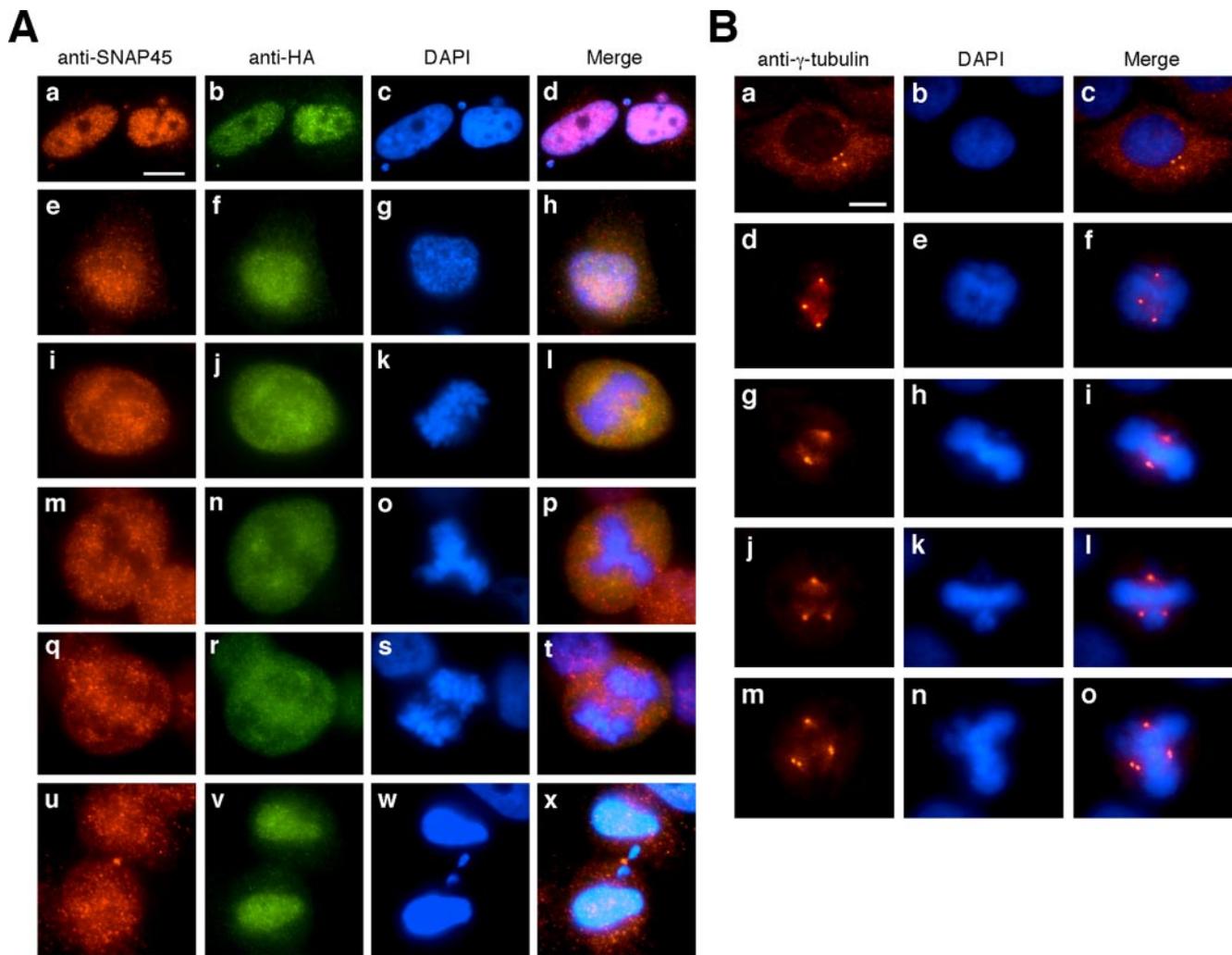


FIGURE 7. **Overexpression of SNAP45 leads to mitotic abnormalities.** *A*, HeLa cells expressing HA-tagged SNAP45 (F1 cell line) were fixed with 2% paraformaldehyde (*panels a–x*) and stained for indirect immunofluorescence with anti-SNAP45 (*red*) and anti-HA (antibody HA.11, Covance) (*green*) antibodies. DNA was stained with DAPI (*blue*). Scale bar, 10  $\mu$ m. *B*, HeLa cells expressing HA-tagged SNAP45 (F1 cell line) were fixed with ice-cold methanol and stained for indirect immunofluorescence with anti- $\gamma$ -tubulin (*red*) antibodies. DNA was stained with DAPI (*blue*). Scale bar, 10  $\mu$ m.

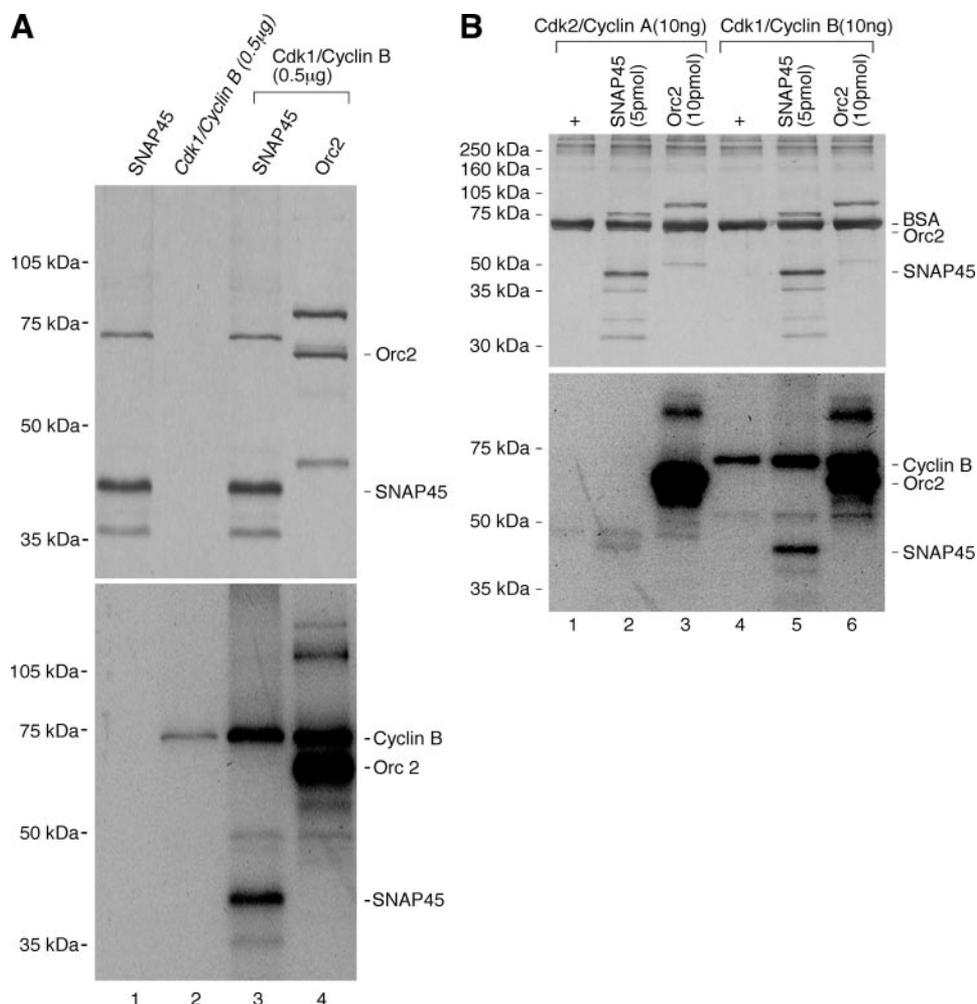
Even though the cells shown in Fig. 6D look relatively normal, 39% of the cells overexpressing SNAP45 displayed abnormalities compared with 8% of cells transfected with the empty vector. Examples of such abnormalities are shown in Fig. 7A, which displays staining with anti-SNAP45 (*red*) and anti-HA (*green*) antibodies, as well as with DAPI. Staining with the anti-SNAP45 antibody gave again a very similar pattern as staining with the anti-HA antibody during all stages of mitosis (Fig. 7A, compare *panels a* and *b*, *e* and *f*, *i* and *j*, *m* and *n*, and *q* and *r*) except in telophase (*panels u* and *v*). Micronuclei were visible in interphase cells (Fig. 7A, *panels a–d*). Problems in chromosome localization were apparent in metaphase cells, probably due at least in part to a multipolar spindle (Fig. 7A, *panels m–p*), and chromosome segregation was defective during anaphase and telophase (*panels q–x*) with lagging chromosomes, probably giving rise to the micronuclei observed in interphase cells.

To examine centrosomes in cells overexpressing SNAP45, we stained with an anti- $\gamma$ -tubulin antibody, as shown in Fig. 7B. This revealed a greatly increased number of multiple aster cells, from 2% in the cell population transfected with the empty vector to 10% in the cell population overexpressing SNAP45. Thus,

overexpression of SNAP45 leads to a series of defects very similar to those observed upon depletion of SNAP45.

*SNAP45 Is a Substrate for Cdk1/Cyclin B in Vitro*—Visual inspection of the SNAP45 amino acid sequence revealed five putative phosphorylation sites for the Cdk1/cyclin B kinase. A consensus phosphorylation site ((S/T)PX(K/R)) (16), TPAR, is present at the very C terminus of the protein. Moreover, four sites matching the minimal consensus sequence ((S/T)P (16)) occur at positions 181 (TP), 222 (SP), 292 (TP), and 306 (SP). We therefore checked whether this kinase could phosphorylate SNAP45 *in vitro*. The *upper* and *lower panels* in Fig. 8A show the proteins included in the reaction as seen by silver stain and radioactive signal, respectively. SNAP45 on its own did not get phosphorylated (Fig. 8A, *lower panel, lane 1*), whereas Cdk1/cyclin B showed autophosphorylation of cyclin B, as expected (*lower panel, lane 2*) (17). When Cdk1/cyclin B was incubated together with either SNAP45 or the positive control Orc2, a known substrate of Cdk1/cyclin B (18), strong radioactive signals corresponding to phosphorylated cyclin B as well as phosphorylated SNAP45 and Orc2, respectively, were visible ((Fig. 8A, *lower panel, lanes 3* and *4*). We then lowered the amount of kinase, included an excess of

## Mitotic Functions of the $SNAP_c$ Subunit $SNAP45$



**FIGURE 8. Cdk1/cyclin B specifically phosphorylates  $SNAP45$  *in vitro*.** A, 5 pmol of bacterially expressed and purified recombinant  $SNAP45$  or Orc2 (as a positive control) were incubated with the indicated amount of Cdk1-cyclin B complex in the presence of [ $\gamma$ - $^{32}$ P]ATP. The *top panel* shows the silver-stained gel and the *bottom panel* an autoradiogram of the same gel. B, Cdk1/cyclin B specifically phosphorylates  $SNAP45$  in the presence of nonspecific competitor BSA. The reactions were carried out with the components indicated *above* the lanes, [ $\gamma$ - $^{32}$ P]ATP, and 0.1 mg/ml of BSA. The *top panel* shows the silver-stained gel and the *bottom panel* an autoradiogram of the same gel.

BSA in the reactions, and tested in addition Cdk2/cyclin A, which is known to phosphorylate Orc2 but presumably not  $SNAP45$ . As shown in Fig. 8B, BSA, which co-migrated with Orc2 as seen by silver staining (*upper panel*), was phosphorylated neither by Cdk2/cyclin A nor by Cdk1/cyclin B (*lower panel, lanes 1 and 4*). Orc2 was phosphorylated by both enzymes (Fig. 8B, *upper and lower panels, lanes 3 and 6*), indicating that both kinase complexes were active. As expected, cyclin B was phosphorylated in the reactions containing Cdk1/cyclin B (Fig. 8B, *lower panel, lanes 4–6*). Unlike Orc2,  $SNAP45$  was only phosphorylated by Cdk1/cyclin B (Fig. 8B, compare *lanes 2 and 5*), and this even in the presence of large amounts of BSA, suggesting that this phosphorylation event is specific. Indeed,  $SNAP45$  was similarly refractive to phosphorylation by Cdk2/cyclin E (see supplemental Fig. S6). Thus,  $SNAP45$  is a specific substrate of Cdk1/cyclin B, at least *in vitro*.

### DISCUSSION

*SNAP45 Localizes to Different Structures during the Cell Cycle and Is Required for Proper Mitotic Progression*— $SNAP_c$ , also called PTF, has been shown to localize together with Oct-1

within the nucleoplasm in Oct-1/PTF/transcription (OPT) domains, which contain transcription factories that may specialize in snRNA gene transcription (19). Here we show that one of the subunits of endogenous  $SNAP_c$ ,  $SNAP45$ , localizes to the centrosomes from metaphase to telophase as well as to the spindle midzone during anaphase and the midbody during telophase. Consistent with this association with structures involved in cell division, we observe major mitotic progression defects when  $SNAP45$  levels are either reduced by siRNA or increased by overexpression of a tagged  $SNAP45$  protein. In both cases, there is an accumulation of cells with  $G_2/M$  DNA contents and defects, including abnormal and multipolar spindles, abnormal chromosome condensation, defective chromosome localization during metaphase and defective segregation, and formation of micronuclei. It is remarkable, then, that it is possible to obtain permanent cell lines overexpressing tagged  $SNAP45$  (20). Such cell lines, although clonal, are characterized during early passages by heterogeneous  $SNAP45$  expression, slow growth curves, and multinucleated cells,<sup>3</sup> suggesting that the overexpression of tagged  $SNAP45$  is not stable and detrimental to the cells. Nevertheless, after the early passages, stable cell lines

overexpressing  $SNAP45$  can be obtained, suggesting that  $SNAP45$  overexpression is tolerated stochastically in some cells that are then positively selected. In much the same way, Mad2 overexpression (or mild down-regulation) is tolerated, leading to aneuploidy and selection of cells with a growth advantage resulting probably from loss of heterozygosity at tumor suppressor loci, whereas loss of Mad2 results in massive chromosome missegregation and mitotic catastrophe (Ref. 21 and references therein).

*SNAP45 Plays a Role Different from Other  $SNAP_c$  Subunits*— $SNAP45$  was first identified as a subunit of  $SNAP_c$ , a complex involved in snRNA gene transcription (9, 22). However, we have not seen  $SNAP_c$  subunits other than  $SNAP45$  localized to centrosomes and mid-bodies during mitosis. Moreover, down-regulation of  $SNAP190$ , which results in concomitant down-regulation of the other  $SNAP_c$  subunits  $SNAP50$  and  $SNAP45$ , results in accumulation of cells with  $G_0/G_1$  DNA content. This

<sup>3</sup> M. Shanmugam and N. Hernandez, unpublished observations.

is consistent with a role of SNAP<sub>c</sub> as a factor involved in transcription of small nuclear RNAs essential for cell metabolism, and indeed consistent with the observation that down-regulation of a SNAP50 homologue in plants prevents efficient cell proliferation (23). In contrast, down-regulation of SNAP45 does not result in obvious down-regulation of SNAP190 or SNAP50 and causes a mitotic rather than a G<sub>0</sub>/G<sub>1</sub> arrest. These observations suggest that SNAP45 may play two different roles in the cell, one in transcription as a subunit of SNAP<sub>c</sub> and the other in mitotic progression as a protein probably outside of the SNAP<sub>c</sub> context.

It is intriguing that like other proteins essential for mitosis, such as NuMA and TPX2 (24–26), SNAP45 has appeared relatively late in evolution as SNAP45-like sequences can be found only in vertebrates. There is a recognizable SNAP<sub>c</sub> in *Drosophila melanogaster*, but the complex is limited to the components present in the minimal functional core of SNAP<sub>c</sub>, mini-SNAP<sub>c</sub> (27), and lacks SNAP45 as well as the SNAP190 C-terminal third that, in human SNAP<sub>c</sub>, associates with SNAP45. Thus, SNAP45 seems to have appeared in vertebrates, both as an elaboration of the SNAP complex, and as a protein required for proper mitosis.

**Human SNAP45 Is Phosphorylated in Vitro by Cdk1/Cyclin B**—Among vertebrates, SNAP45 is quite conserved (for example, 65% identities between man and dog, and 25% identities between man and zebrafish SNAP45). The consensus Cdk1/cyclin B phosphorylation site at the very C terminus is, however, only present in some species (*Macaca mulatta* and *Macaca fascicularis*, *Canis familiaris*, and *Mus musculus*), although all proteins contain minimal Cdk1/cyclin B putative phosphorylation sites, and may thus also be phosphorylated by this kinase but at different sites. We show that human SNAP45 is a good substrate for Cdk1/cyclin B *in vitro*, suggesting that it might also be phosphorylated by this kinase *in vivo*. Phosphorylation by Cdk1/cyclin B often serves to disrupt protein-protein interactions, and thus one might imagine that it might release SNAP45 from SNAP<sub>c</sub>. On the other hand, SNAP45 phosphorylation by Cdk1/cyclin B may more directly regulate its role during mitosis, for example by allowing its recruitment to centrosomes, in the same way that Cdk1/cyclin B phosphorylation of Eg5 allows its recruitment to centrosomes and spindle microtubules (28, 29).

**Alteration of SNAP45 Levels Causes Multiple Mitotic Defects**—For many proteins involved in mitosis, up- and down-regulation causes a wide variety of effects. For example, subunits of Orc such as Orc2 and Orc6 localize to centrosomes, centromeres, and heterochromatin in the first case, and kinetochores and mid-bodies in the second case. Down-regulation of these proteins by siRNA causes not only defects in S phase but also multiple centrosomes, abnormally condensed chromosomes, failed chromosome congression, and, in the second case, multinucleated cells (30). As another example, BRCA1 localizes to centrosomes (31, 32), and its down-regulation causes amplification and fragmentation of centrosomes in cell lines derived from mammary tissue (33, 34) and defective chromosome condensation and segregation in other cell lines (35). Like for Orc2, Orc6, Brca1, and a number of other proteins, disrupting the normal levels of SNAP45 results in a number of

mitotic defects, which suggests an involvement of SNAP45 in several processes, including the centrosome cycle, the alignment of chromosomes at the metaphase plate, and chromosomes condensation and segregation. Finally, the localization of SNAP45 in mid-bodies suggests a role in cytokinesis, a possibility supported by an observed increase of multinucleated cells in cell lines overexpressing SNAP45 (data not shown).

The defects observed upon both up-regulation and down-regulation of SNAP45 include defective chromosome segregation and formation of micronuclei. In some cells this may lead to aneuploidy, which itself can be both oncogenic and tumor-suppressing (21, 36). In the case of SNAP45, severe depletion is likely to lead to cell death as a result of both defective mitosis and defective snRNA gene transcription. Indeed, we observe activation of pro-caspase 9 at late times after transfection of siRNAs directed against SNAP45. However, all our experiments were performed with the transformed HeLa cell line. Depletion of polo-like kinase 1 leads to cell death in p53-deficient cancer cells but has little effect in nontransformed cells, making polo-like kinase 1 a potential target for cancer therapy (37). It will be interesting to determine whether mild depletion of SNAP45, which may still maintain enough snRNA gene transcription, is tolerated better by normal cells as compared with transformed cells.

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## Mitotic Functions of the SNAP $\zeta$ Subunit SNAP45

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