SCAPER, a novel cyclin A–interacting protein that regulates cell cycle progression

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Cyclin A/Cdk2 plays an important role during S and G2/M phases of the eukaryotic cell cycle, but the mechanisms by which it regulates cell cycle events are not fully understood. We have biochemically purified and identified SCAPER, a novel protein that specifically interacts with cyclin A/Cdk2 in vivo. Its expression is cell cycle independent, and it associates with cyclin A/Cdk2 at multiple phases of the cell cycle. SCAPER localizes primarily to the endoplasmic reticulum. Ectopic expression of SCAPER sequesters cyclin A from the nucleus and results specifically in an accumulation of cells in M phase of the cell cycle. RNAi-mediated depletion of SCAPER decreases the cytoplasmic pool of cyclin A and delays the G1/S phase transition upon cell cycle re-entry from quiescence. We propose that SCAPER represents a novel cyclin A/Cdk2 regulatory protein that transiently maintains this kinase in the cytoplasm. SCAPER could play a role in distinguishing S phase– from M phase–specific functions of cyclin A/Cdk2.

Introduction

Cyclin-dependent kinases (Cdks) play a key role in mammalian cell cycle regulation by ensuring that cell cycle events proceed in a stepwise fashion and produce two identical daughter cells. Several cyclin/Cdk complexes have been implicated in cell cycle control (Sherr and Roberts, 2004). In mammalian cells, entry into mitosis is governed by cyclin A/Cdk1 and cyclin B/Cdk1. Another Cdk activity, cyclin A/Cdk2, is required for both DNA synthesis and mitosis (Pagano et al., 1992). Cyclin A/Cdk2 activity is first evident in late G1, and it persists through S and G2 phase until prometaphase (Furuno et al., 1999; den Elzen and Pines, 2001; Geley et al., 2001). To date, two known isoforms of mammalian cyclin A (cyclin A1 and A2) are present. Although cyclin A1 is restricted to male germ cells, cyclin A2 (hereafter referred to as cyclin A) is widely expressed in both germ cells and somatic tissues (Howe et al., 1995; Sweeney et al., 1996). Cyclin A–deficient mice die during early embryogenesis (Murphy, 1999), demonstrating that cyclin A is an essential gene.

The requirements for cyclin A in both replication and mitosis have not been fully elucidated. A number of experimental approaches have suggested potential mechanisms whereby cyclin A/Cdk2 complexes regulate the G1/S transition. Overexpression of cyclin A accelerated entry of mammalian cells into S phase, indicating that this cyclin could be a limiting factor needed to trigger replication (Resnitzky et al., 1995). Conversely, microinjection of anti-cyclin A antibodies or production of antisense RNA in G1 phase cells prevented entry into S phase (Girard et al., 1991). It has been proposed that cyclin A/Cdk2 is required for the initiation of replication, activation of pre-existing replication complexes, and/or prevention of rereplication (Saha et al., 1998; Petersen et al., 1999; Coverley et al., 2002). In support of these mechanisms, cyclin A has been found to associate with DNA replication foci in human cells, and it has been shown to bind to and phosphorylate proteins in the origin recognition complex in *Xenopus* extracts (Cardoso et al., 1993; Romanowski et al., 2000). Cyclin A has also been shown to associate with and phosphorylate Cdc6, provoking its nuclear export and degradation and thereby preventing rereplication (Saha et al., 1998; Petersen et al., 1999).

The mechanisms by which cyclin A promotes entry into, and progression through, mitosis are also unclear. This is likely due, at least in part, to the functional overlap with another mitotic kinase, cyclin B/Cdk1. Nevertheless, it is clear that cyclin A plays an essential role in mitotic entry because microinjection of anti-cyclin A antibodies into G2 phase cells can prevent progression into M phase (Pagano et al., 1992). Microinjection of recombinant cyclin A/Cdk2 into human G2 (but not S phase) cells accelerated entry into mitosis, suggesting that this kinase may be

a rate-limiting factor for the G2/M transition (Furuno et al., 1999). Interestingly, cyclin A/Cdk2 was also required to progress through mitosis until the middle of prophase because the inhibition of this kinase with a Cdk inhibitor caused early or mid-prophase cells to return to interphase (Furuno et al., 1999). Cyclin A is destroyed by the anaphase-promoting complex in pro-metaphase, and mutants lacking a destruction box arrest in anaphase (Geley et al., 2001). In light of these experiments and others, it will be critical to determine the key substrates of this kinase, how they enable progression through replication and mitosis, and whether these targets are specific to cyclin A/Cdk complexes.

In addition to its role in activating Cdks, the cyclin component is critical for directing kinase activity to particular compartments. Cyclins are directed to their substrates through signals that regulate both subcellular localization and targeting to a specific protein binding site (the so-called RXL or cyclin-binding motif) (Zhu et al., 1995; Adams et al., 1996; Chen et al., 1996). Cyclin A had been shown previously to be predominantly nuclear by immunofluorescence (Pines and Hunter, 1991). Yet it is clear that cyclin A/Cdk2 phosphorylates both nuclear and cytoplasmic targets, including those involved in centrosome duplication (Meraldi et al., 1999). Interestingly, recent data suggest that the cyclin A/Cdk complex is not statically maintained in the nucleus. Rather, it shuttles between nucleus and cytoplasm, consistent with its ability to modify targets in both compartments (Jackman et al., 2002). These and other studies also suggested that Cdk2 was required in vitro and in vivo for nuclear import of cyclin A (Maridor et al., 1993). However, the requirements for cyclin A nuclear export have not been defined.

In an effort to understand in greater detail how cyclin A is regulated, we have performed a biochemical screen for proteins that interact with cyclin A in human cells. Our efforts have led to the identification of SCAPER, a novel protein that specifically interacts with cyclin A/Cdk2 in vivo through a cyclin-binding motif. Although a small portion is associated with the nucleus, SCAPER localizes primarily to the endoplasmic reticulum (ER), and its expression is relatively constant throughout the cell cycle. It associates with cyclin A at multiple stages of the cell cycle. Ectopic expression of SCAPER sequesters cyclin A from the nucleus and delays cell cycle progression in M phase. Furthermore, ablation of SCAPER by RNAi decreases the pool of cyclin A in the cytoplasm (manifested as a membrane-bound complex), resulting in delayed progression into S phase from quiescence (G0) in response to mitogens. Our data suggest that the isolation of SCAPER may allow us to dissect the S and M phase functions of the cyclin A/Cdk2 kinase. Further, SCAPER binding to cyclin A may represent a mechanism for retaining cyclin A transiently in the cytoplasm and directing the kinase to specific substrates that must be phosphorylated in that compartment before S phase entry.

Results

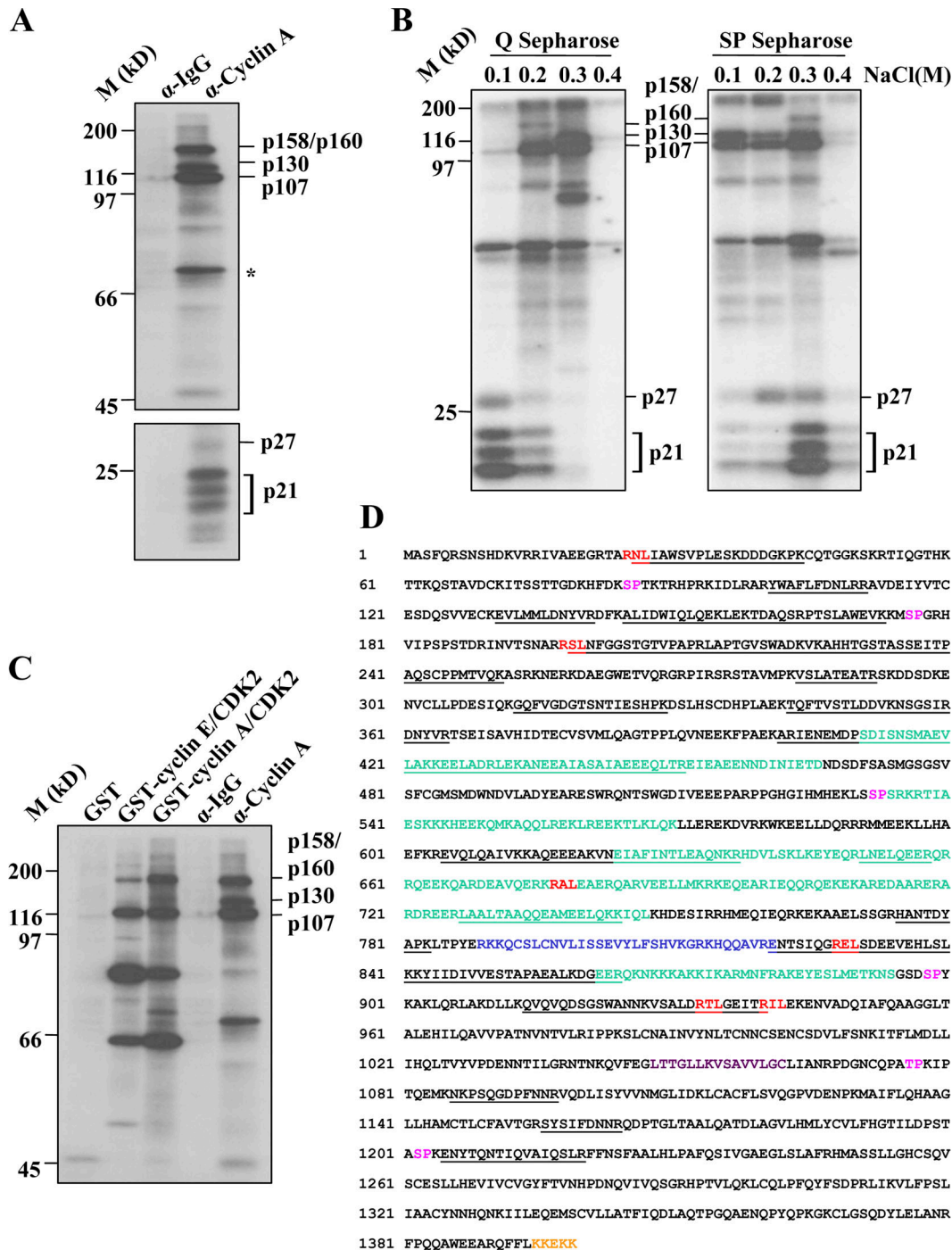### Purification of SCAPER, a cyclin A/Cdk2-interacting protein

To search for cyclin A/Cdk2-interacting proteins, we took advantage of the observation that cyclins often exhibit stable binding to substrates and regulators through dedicated docking sites and that the resulting associated proteins can be subsequently phosphorylated in vitro. We biochemically isolated proteins that associate with cyclin A in vivo using anti-cyclin A immunoaffinity purification. Purified proteins were subjected to in vitro kinase assays and separated by SDS-PAGE, revealing a series of phosphorylated species (Fig. 1 A). Given that the Kip/Cip family of kinase inhibitors, p21 and p27, and the pRB family proteins, p107 and p130, have been shown to associate with cyclin A in vivo, we used a combination of immunodepletion, Western blotting, and kinase assay to verify that they were indeed present in this collection of polypeptides (Fig. 1 A and unpublished data). These results substantiate our approach and suggest that physiological cyclin A/Cdk2-interacting proteins can be identified using this strategy.

In addition to the expected complement of proteins described above, we detected a major phosphorylated polypeptide of 158/160 kD (p158/p160) that did not correspond to known cyclin A–interacting proteins. To further validate the interaction between cyclin A and p158/p160, we fractionated whole cell extracts using Q- and SP-Sepharose ion exchange columns and then performed immunopurification with anti-cyclin A antibody and kinase assays. p107 and p130 appeared in multiple fractions (Fig. 1 B), attesting to their heterogeneous representation in multiple, distinct complexes (Woo et al., 1997). In contrast, p158/p160 was detected in a single fraction on either ion exchange column, suggesting the existence of stable, specific cyclin A/Cdk2 complexes. To further test the notion that p158/p160 could be isolated by an independent method that also relied on affinity for cyclin A/Cdk2, we chromatographed human cell extracts over a matrix containing recombinant GST-cyclin A/Cdk2 and performed kinase assays. GST-cyclin A/Cdk2, but not unfused GST protein, interacted with a polypeptide indistinguishable in mobility from p158/p160 (Fig. 1 C). GST-cyclin E/Cdk2 also bound p158/p160, albeit to a lesser extent, in this in vitro experiment.

Purification and identification of SCAPER

To identify p158/p160, we performed a large-scale purification of the protein from human cell extracts using resin containing recombinant GST-cyclin A/Cdk2 protein. The 158/160-kD band was subjected to mass spectrometric sequencing. This analysis revealed that a large number of peptides belonged to two uncharacterized proteins with similar molecular masses. One of these polypeptides has a theoretical molecular weight of 158 kD and encoded a putative zinc finger motif (sequenced previously as a cDNA designated ZNF291, GenBank/EMBL/DDBJ accession no. AF242528; Fig. 1 D). We termed this polypeptide SCAPER (S phase cyclin A–associated protein residing in the endoplasmic reticulum) in light of our findings described below. SCAPER shows no considerable homology with known human proteins. A motif search identified a putative C2H2-type zinc finger motif, a putative transmembrane domain, an ER retrieval signal at the C terminus, four coiled-coil domains, six potential RXL motifs that might confer binding to cyclins, and six consensus Cdk phosphorylation sites (Fig. 1 D). We note the existence of mouse, rat, bovine, and chimpanzee SCAPER orthologues.

Figure 1. Identification of SCAPER using immunoaffinity purification. (A) Proteins interacting with cyclin A were identified via immunopurification with cyclin A antibody and kinase assay. Control IgG was used as a control. p21, p27, p107, and p130 were identified by immunoprecipitation and Western blotting as well as immunodepletion. The asterisk indicates an unidentified polypeptide. (B) p158/p160 complexes with cyclin A were fractionated by Q and SP Sepharose ion exchange chromatography and eluted with the indicated concentrations of salt. Complexes were detected as in A. (C) Proteins purified with matrices containing the various GST-tagged complexes were identified by kinase assays and compared with eluates from anti-cyclin A immuno-purification as in A. (D) The complete amino acid sequence of SCAPER. Possible RXL motifs and Cdk phosphorylation sites are highlighted in red and pink, respectively. Putative coiled-coil domains are highlighted in cyan; zinc finger motif in blue; ER retrieval signal in orange; and transmembrane motif in purple. Peptides sequenced by mass spectrometry are underlined.

We amplified the coding region, inserted the resulting product into an expression vector with a Flag tag, and expressed the protein in human 293T cells. Flag-SCAPER was purified and could be phosphorylated in vitro with recombinant cyclin A/Cdk2. The resulting phosphoprotein co-migrated with endogenous SCAPER (Fig. 2 A), suggesting that we had amplified the full-length protein.

We probed Northern blots derived from human tissue and found that SCAPER is ubiquitously expressed in each of the human tissues we examined, with highest transcript levels in
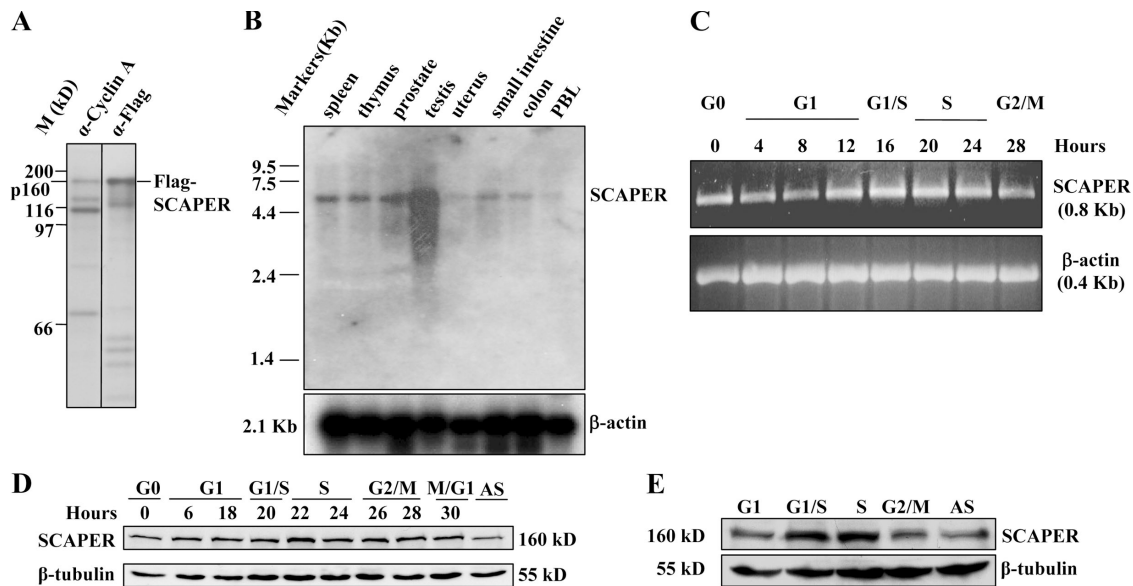
Figure 2. **mRNA and protein expression of SCAPER.** (A) Full-length SCAPER (lane 2) was cloned as a Flag-tagged fusion protein, expressed in 293 cells, immunoprecipitated with anti-Flag beads, and phosphorylated with recombinant GST-cyclin A/Cdk2 in vitro. Endogenous cyclin A/Cdk2/SCAPER complex isolated by immunopurification with cyclin A antibody was used as a control (lane 1). (B) Expression of SCAPER in human tissues. mRNA from different human tissues was probed with a SCAPER cDNA, and β-actin was used as a control. PBL: peripheral blood lymphocytes. (C) SCAPER mRNA expression across the cell cycle. T98G cells were synchronized by serum deprivation and restimulation with FBS. Semi-quantitative RT-PCR was used to measure SCAPER mRNA levels, and β-actin was used as a loading and normalization control. (D and E) Western blotting of endogenous SCAPER. (D) T98G cells were synchronized by serum deprivation and restimulation. β-Tubulin was used as a loading control. (E) U2OS cells were synchronized to G1, G1/S, S, and G2/M with drugs.

testis (Fig. 2 B). Next, we determined whether SCAPER was expressed in a cell cycle–dependent manner. We synchronized human T98G cells by means of serum deprivation and restimulation and prepared total RNA from cells at each stage. Semi-quantitative RT-PCR revealed that SCAPER was expressed at each stage of the cell cycle, although the gene was expressed at somewhat higher levels in late G1 and S phase (Fig. 2 C). It is worth noting that SCAPER is expressed in G0 cells. Northern blot analysis of total RNA from synchronized T98G cells revealed a transcript of the expected size and confirmed the results we obtained with RT-PCR (unpublished data).

To examine SCAPER protein levels, we produced and affinity purified a polyclonal antibody against an internal fragment of SCAPER located close to the N terminus. SCAPER was the predominant polypeptide visualized by Western blotting of crude cell lysates (Fig. 2, D and E), and it was expressed in every cell line we have tested, including immortal (T98G, U2OS, HeLa, 293T, Saos2) and normal diploid (IMR90, WI38) human cell lines, and mouse myoblast C2C12 cells (unpublished data). We examined SCAPER protein levels as cells progress from G0 to S phase and in cells progressing from the G1/S transition. Consistent with our analysis of RNA, SCAPER is expressed at relatively constant levels throughout the cell cycle, including G0, although it was slightly elevated in early S phase (Fig. 2, D and E).
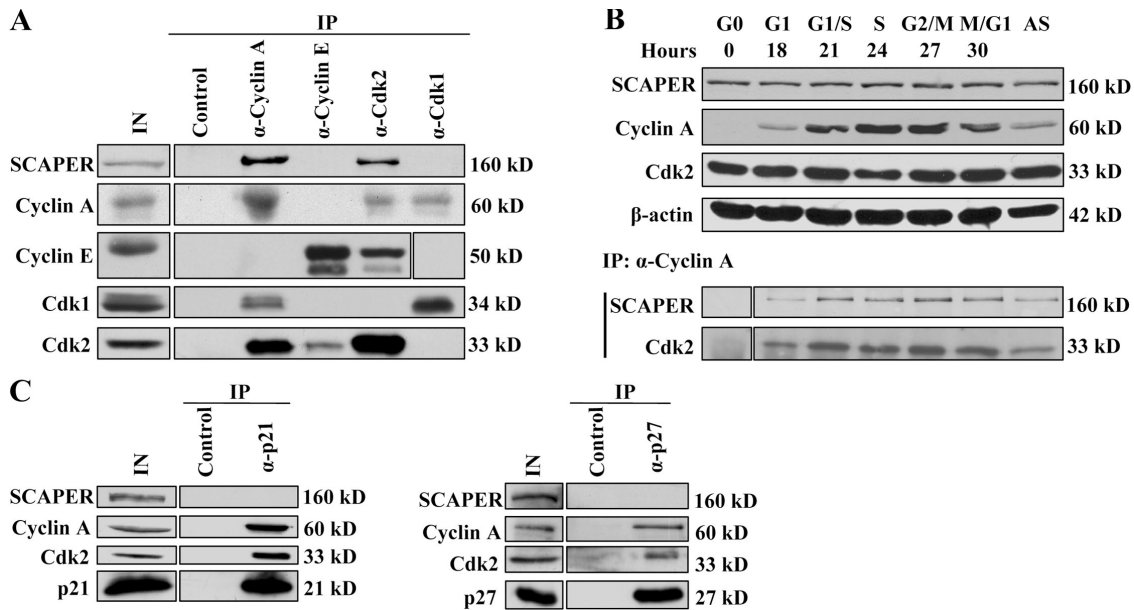
SCAPER specifically interacts with cyclin A/Cdk2 in vivo

Our initial identification of SCAPER was based on the observation that it stably interacts with cyclin A/Cdk2. Given that Cdk substrates are generally capable of associating with multiple cyclin/Cdk complexes, we asked whether SCAPER also interacts with other cyclins or Cdks in vivo. Endogenous SCAPER could be immunoprecipitated from cell extracts with anti-cyclin A or anti-Cdk2 antibody, but not with anti-cyclin E or anti-Cdk1 antibody (Fig. 3 A). This suggests that SCAPER specifically interacts with cyclin A/Cdk2, but not with cyclin A/Cdk1, cyclin B/Cdk1, or cyclin E/Cdk2, in vivo, within the detection limits of our Western blot analysis. This in vivo interaction was also detected using normal human diploid fibroblasts (IMR90) and mouse myoblasts (unpublished data). Similar results were obtained when we transiently transfected 293 cells with a Flag-SCAPER expression vector, immunoprecipitated the protein with anti-Flag antibodies, and performed Western blotting with antibodies against different cyclins and Cdks. We observed coimmunoprecipitation of Flag-SCAPER with cyclin A and Cdk2, but not with cyclin E or Cdk1 (Fig. 4 B, lane 9). Next, we investigated the interaction between SCAPER and cyclin A at different cell cycle stages in T98G cells rendered quiescent and restimulated to enter the cell cycle. Like SCAPER, Cdk2 levels were relatively constant throughout the cell cycle (Fig. 3 B). In contrast, cyclin A expression peaks in S/G2 phase, after which it decreases (Fig. 3 B), as expected. Interestingly, cyclin A associates with SCAPER whenever it is expressed (Fig. 3 B). Collectively, our results demonstrate that SCAPER specifically interacts with cyclin A/Cdk2 at multiple phases of the cell cycle, including S and G2/M.

We investigated whether SCAPER/cyclin A/Cdk2 complexes also contained members of the Kip/Cip family of Cdk inhibitory proteins and/or F-box family proteins. Strikingly, we

Figure 3. **SCAPER specifically interacts with cyclin A/Cdk2 and does not interact with p21 or p27 in vivo.** (A) Western blotting of endogenous SCAPER, cyclin A, cyclin E, Cdk1, and Cdk2 after immunoprecipitation with the various antibodies indicated or control (anti-calnexin) antibody from 293T cell extracts. IN represents input. The size of cyclin E in the input lane appears to be slightly larger than the one detected in the IP lanes. This phenomenon occurred sporadically and is likely ascribed to fluctuations in electrophoresis and/or transfer conditions. (B) SCAPER interaction with cyclin A across the cell cycle. T98G cells were synchronized by serum starvation and restimulated. (Top) Western blotting of endogenous SCAPER, cyclin A, and Cdk2. Actin was used as a loading control. (Bottom) Western blotting of endogenous SCAPER and Cdk2 after immunoprecipitation with an anti-cyclin A antibody. (C) Western blotting of endogenous SCAPER, cyclin A, Cdk2, p21, or p27 after immunoprecipitation with control (anti-calnexin), p21, or p27 antibody from U2OS cell extracts. IN represents input.

found that immunoprecipitates of p21 and p27 contained cyclin A and Cdk2, as expected, but not SCAPER (Fig. 3 C). Thus, the SCAPER/cyclin A/Cdk2 complex is separable from the pools of cyclin A/Cdk2/p21 and cyclin A/Cdk2/p27, and this finding is consistent with our immunodepletion experiments (unpublished data). Furthermore, we note that immunoprecipitates of another cyclin A–interacting protein, Skp2, do not contain SCAPER (unpublished data).

A single cyclin-binding motif at the N-terminal region of SCAPER is responsible for cyclin A/Cdk2 interaction

To identify the region in SCAPER that confers binding to cyclin A/Cdk2, N-terminal (residues 1–717; SCAPER-N) and C-terminal (residues 717–1399; SCAPER-C) fragments of SCAPER were fused to a Flag tag and expressed in both bacteria and mammalian cells (Fig. 4 A). Subsequent immunoprecipitation of transfected human 293T cells revealed that cyclin A and Cdk2 strongly associated with Flag-SCAPER-N, but only weak interactions were evident with Flag-SCAPER-C (Fig. 4 B). Identical results were obtained when bacterially expressed Flag-SCAPER-N and Flag-SCAPER-C were incubated with recombinant GST-cyclin A/Cdk2 (Fig. 4 C). Thus, the N-terminal region of SCAPER is responsible for cyclin A/Cdk2 binding, and the interaction between SCAPER and this kinase is direct. Further, kinase assays revealed that cyclin A/Cdk2 phosphorylates Flag-SCAPER-N but not Flag-SCAPER-C (Fig. 4 D), suggesting that cyclin A/Cdk2 can both bind and phosphorylate the N-terminal half of SCAPER. We note that the addition of excessive amounts

of exogenous SCAPER did not substantially inhibit cyclin A/Cdk2 kinase activity, suggesting that this fragment of SCAPER is not a Cdk inhibitor (Fig. 4 D and unpublished data).

Previous work revealed that RXL motifs in Cdk-interacting proteins are responsible for stable interactions with cyclin/Cdks (Zhu et al., 1995; Adams et al., 1996; Chen et al., 1996), and we examined the requirement for this motif in SCAPER. The N-terminal portion of SCAPER confers cyclin A/Cdk2 binding (Fig. 4, B and C), and given that there are three cyclin-binding motifs in SCAPER-N, we mutated each of them in the context of full-length SCAPER to generate mutants mA, mB, and mC (Fig. 4 A). Each mutant was expressed in 293T cells. Immunoprecipitation and Western blotting clearly show that mutants mA and mC retain the ability to bind to cyclin A/Cdk2 to the same extent as wild-type SCAPER (Fig. 4 B). In contrast, mB is unable to associate with cyclin A/Cdk2 (Fig. 4 B), indicating that this region constitutes a bona fide cyclin-binding motif.

SCAPER is a perinuclear protein localized to the nucleus and primarily to the ER

To biochemically examine the subcellular localization of SCAPER, we fractionated cell extracts into nuclear, cytosolic, and membrane compartments and verified enrichment of each fraction using several well-established markers (Fig. 5 A). Cyclin A is found predominantly in the nucleus, whereas Cdk2 is found in both the nucleus and the cytosol, in agreement with earlier reports (Pines and Hunter, 1991; Bresnahan et al., 1996; Jackman et al., 2002). Interestingly, SCAPER is most enriched in the membrane fraction, similar to an ER membrane protein
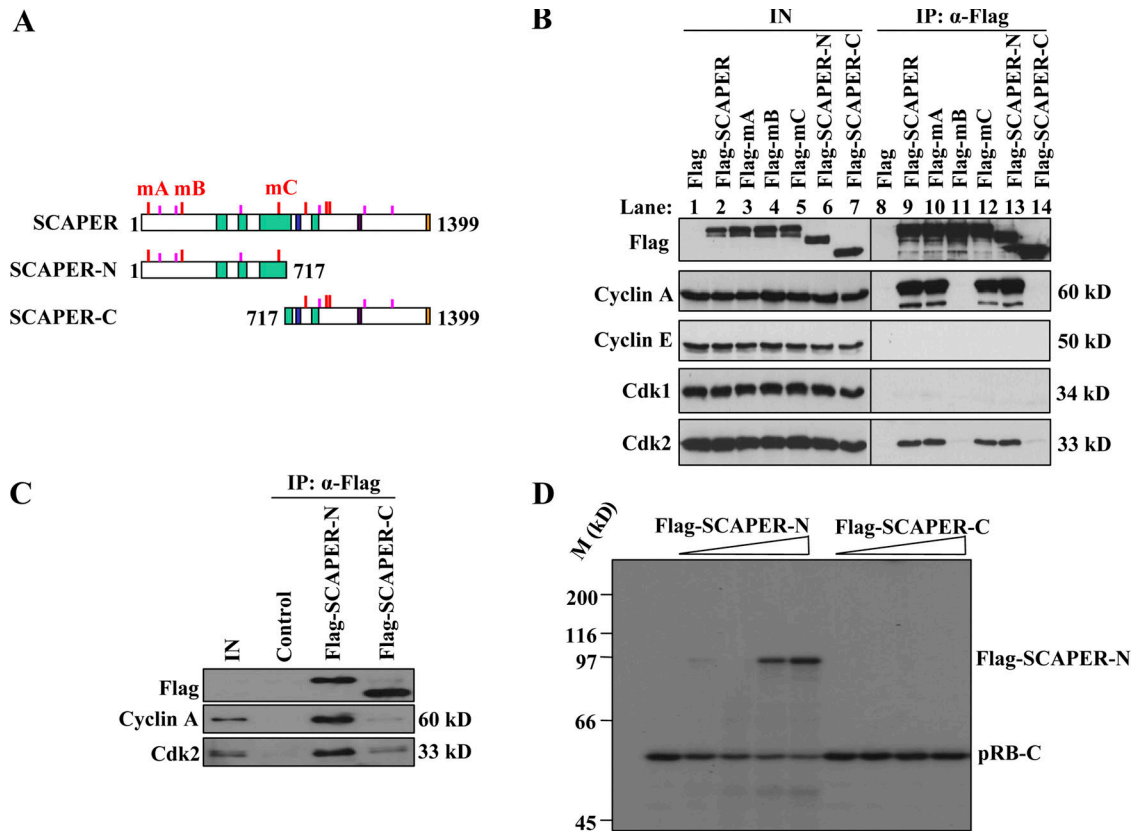
Figure 4. **A single RXL motif near the N-terminal of SCAPER binds specifically to cyclin A/Cdk2.** (A) Schematic illustration of SCAPER and mutants. All constructs were fused to an N-terminal Flag tag. Amino acid residue numbers are stated. (B) Flag-tagged SCAPER mutants were expressed in 293 cells, immunoprecipitated with anti-Flag antibodies, and immunoblotted with antibodies against cyclin A, cyclin E, Cdk2, and Cdk1. (C) Flag-tagged SCAPER-N, but not SCAPER-C, forms a complex with cyclin A/Cdk2 in vitro. Recombinant SCAPER deletion mutants were mixed with this kinase and purified via the epitope tag on SCAPER. Antibodies used for immunoprecipitation and immunoblotting are indicated. (D) Flag-SCAPER-N but not Flag-SCAPER-C is efficiently phosphorylated by cyclin A/Cdk2.

calnexin, although a portion was also detectable in the nuclear fraction (Fig. 5 A). This is in contrast to a Golgi resident protein giantin, which is located exclusively in the membrane fraction. Further, cyclin A resident in these membrane fractions was associated with SCAPER (Fig. 5 B). We estimate, based on our subcellular fractionation and RNAi experiments (see Fig. 8 B), that ~10–20% of the total cellular cyclin A is bound to SCAPER. Given that the ER is contiguous with the nuclear envelope and that most rough ER membrane proteins are present in both the rough ER and the nuclear envelope (Klopfenstein et al., 2001), these data could suggest that SCAPER is a component of the ER.

Next, we used indirect immunofluorescence to further investigate the subcellular localization of SCAPER. Endogenous SCAPER was associated mostly with perinuclear and reticular structures (Fig. 5 C), with a small but detectable portion localized to the nucleus. Use of a second affinity-purified anti-SCAPER antibody directed against a different epitope gave similar results (unpublished data). We examined the localization of recombinant SCAPER by transfecting Flag-tagged SCAPER into U2OS cells, and indistinguishable results were obtained (Fig. 5 C). Comparable findings were obtained using multiple human cell lines. To more precisely localize SCAPER within the perinuclear region, we performed colocalization experiments with GFP-tagged mannosidase II and GFP-tagged sec61β in the absence or presence

of nocodazole. Mannosidase II is a luminal protein found in the medial compartment of the Golgi. Its staining did not substantially overlap with that of SCAPER (Fig. 5 D). On the other hand, sec61β, a subunit of the heteromeric sec61 translocation complex found in the membrane of the ER, exhibited substantial overlap with SCAPER (Fig. 5 D), indicating that SCAPER may localize to the ER. To test this idea further, we treated cells with a low dose of nocodazole, which depolymerizes microtubules and causes the Golgi to break down into small fragments containing mannosidase II that are dispersed throughout the cytoplasm (Fig. 5 D). In contrast, ER proteins such as sec61β are not affected by nocodazole, and likewise, the distribution of SCAPER was not substantially altered by such treatment (Fig. 5 D). Use of a different drug, brefeldin A, which induces relocalization of Golgi proteins to the ER, has no effect on SCAPER localization (unpublished data). Collectively, subcellular fractionation and the immunofluorescence experiments confirm that SCAPER is an integral component of the ER.

Ectopic expression of SCAPER alters cyclin A localization and affects M phase progression

To study the effect of SCAPER expression on endogenous cyclin A, we ectopically expressed Flag-SCAPER in human cells.
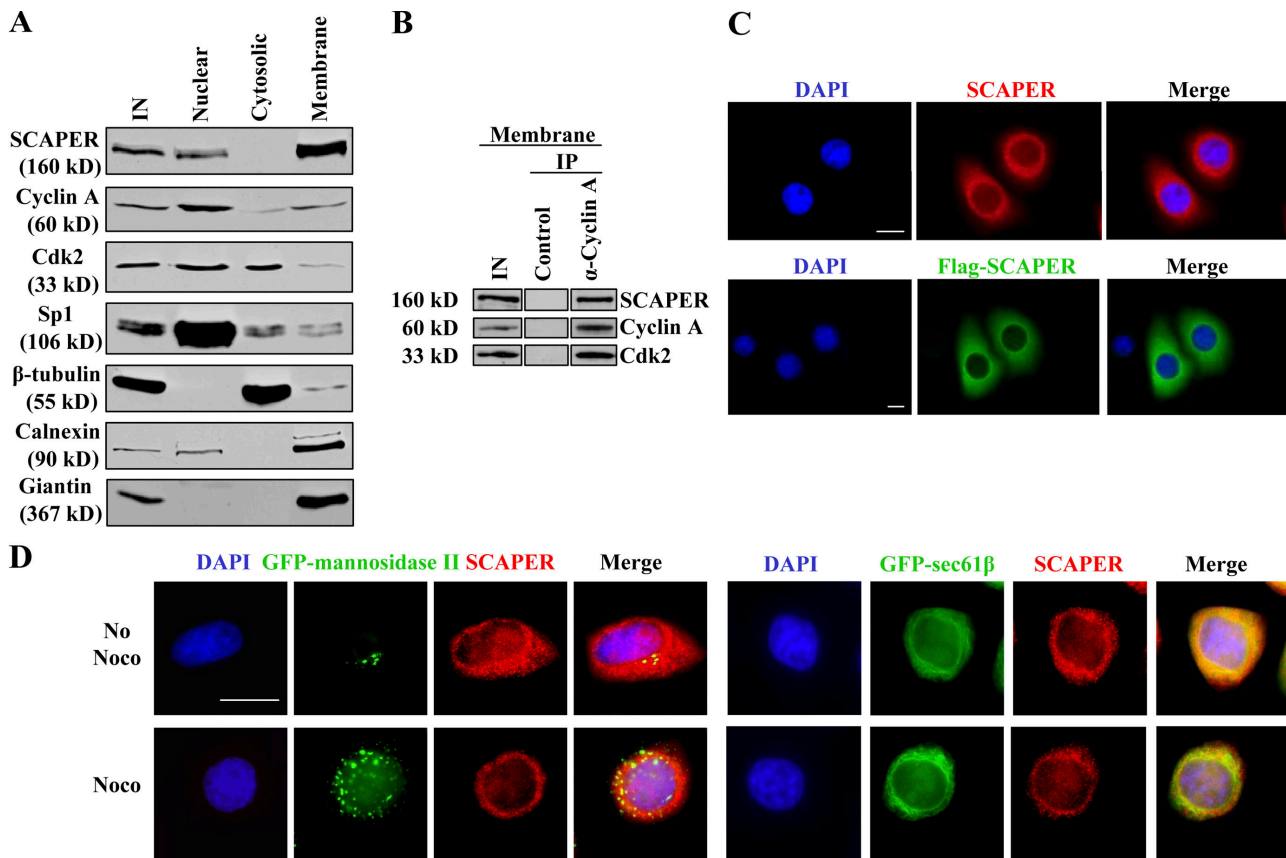
Figure 5. **Subcellular localization of SCAPER.** (A) Subcellular fractionation in U2OS cells. 50 μg of proteins from nuclear, cytosolic, and membrane components were resolved by SDS-PAGE and blotted with the antibodies indicated. IN represents whole cell extract. (B) Western blotting of endogenous SCAPER, cyclin A, and Cdk2 after immunoprecipitation with control (anti-Flag) or anti-cyclin A antibody using the membrane fraction from subcellular fractionation. IN represents input. (C) U2OS cells (top row) or U2OS cells expressing Flag-SCAPER (bottom row) were fixed and incubated with affinity-purified anti-SCAPER antibody (red, top row) or anti-Flag antibody (green, bottom row), and DAPI. Bar, 10 μm. (D) U2OS cells transfected with either GFP-mannosidase II or GFP-sec61β were treated with or without 10 μM nocodazole for 3 h, fixed, and incubated with affinity-purified anti-SCAPER antibody (red) and DAPI. Bar, 10 μm.

Immunofluorescence studies indicated that cyclin A localizes primarily to the nucleus, as expected (Fig. 6 A). However, when Flag-SCAPER was ectopically expressed, endogenous cyclin A was diffusely distributed throughout the cell. A substantial fraction of cyclin A was sequestered outside of the nucleus in at least 70% of Flag-SCAPER–expressing cells (Fig. 6 A). During mitosis, the pattern of cyclin A in Flag-SCAPER–expressing cells looked indistinguishable from control cells (unpublished data). Interestingly, the Flag-SCAPER-N, mA, and mC SCAPER mutants, each of which retains an intact RXL motif (site B), potently sequestered cyclin A in the cytoplasm (Fig. 6 A and unpublished data). In sharp contrast, Flag-SCAPER-C and mutant mB, which lacks this site, were unable to cause cyclin A relocation (Fig. 6 A and unpublished data). Similar results were obtained with other human cell lines. These data strongly suggest that the change in cyclin A localization was mediated by SCAPER binding to cyclin A in the cytoplasm. Thus, the expression of SCAPER can dominantly interfere with cyclin A localization.

If SCAPER specifically associates with cyclin A/Cdk2, and cyclin A/Cdk2 is required at distinct stages of the cell cycle, ectopic expression of SCAPER might be expected to affect cell cycle distribution. We transiently expressed Flag-tagged SCAPER and several mutants in human cells and examined their cell cycle distribution at different intervals after transfection using flow cytometry. SCAPER expression initially caused a modest increase in the S phase population that eventually led to a prominent G2/M accumulation 72 h after transfection (Fig. 6, B and C). To pinpoint the stage at which the arrest occurred, we compared the mitotic index of transfected versus untransfected cells by visualizing cells that exhibit phosphorylation of serine 10 of histone H3, a well-established marker of mitosis (Hendzel et al., 1997). Microscopic examination revealed a fourfold enrichment of mitotic cells expressing Flag-SCAPER (13.3 ± 3.6%) as compared with control mitotic cells (2.8 ± 0.7%) (Fig. 6 D). DAPI staining revealed that these cells had condensed chromosomes. To more precisely determine whether this enrichment in mitotic cells stemmed from a cell cycle block or a delay, we blocked transfected cells at the G1/S phase border with hydroxyurea (HU). After treatment with HU, the mitotic index of control (0.5 ± 0.1%) and SCAPER-expressing cells (0.7 ± 0.1%) did not differ substantially (Fig. 6 D), suggesting that SCAPER overexpression results in an M phase delay rather than an arrest. Furthermore, the proportion of prophase cells in SCAPER-expressing mitotic cells was slightly elevated compared with that in control mitotic cells, suggesting the delay
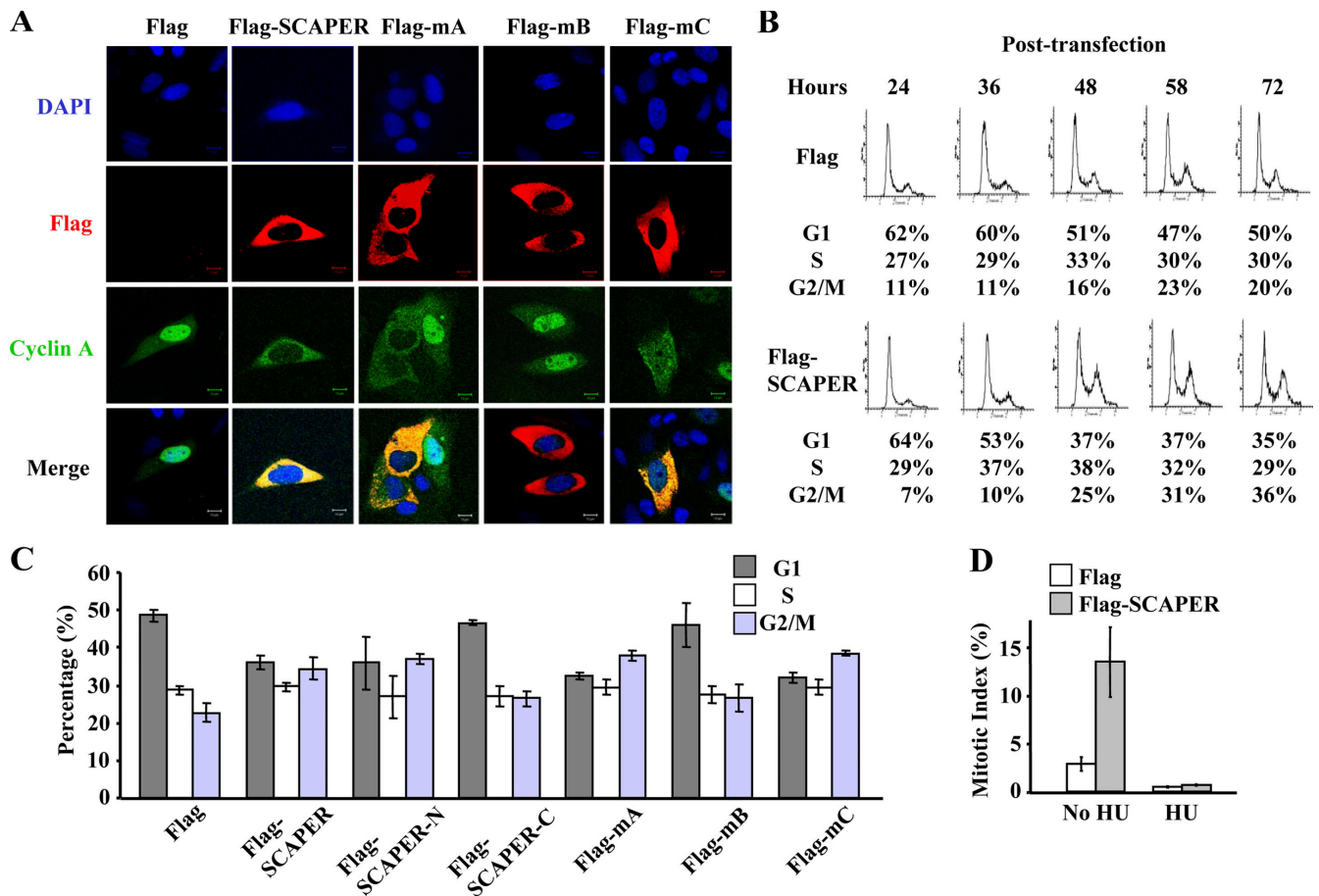
Figure 6. **Expression of SCAPER sequesters cyclin A in the cytoplasm and causes accumulation of cells in M phase.** (A) Saos2 cells were transiently transfected with Flag, Flag-SCAPER, Flag-mA, Flag-mB, or Flag-mC. Cells were fixed and stained with anti-Flag, anti-cyclin A antibodies, and DAPI. Bar, 10 μm. (B) The effect of SCAPER on Saos2 cell cycle distribution. Saos2 cells were transiently transfected with pCMV-CD20 and either Flag or Flag-SCAPER. Cells were harvested and analyzed by flow cytometry. FACS profiles were gated for CD20 fluorescence. (C) The effect of SCAPER deletion (SCAPER-N and SCAPER-C) and RXL (mA, mB, and mC) mutants on cell cycle distribution. Saos2 cells were transiently transfected with pCMV-CD20 and either Flag, Flag-SCAPER, Flag-SCAPER-N, Flag-SCAPER-C, Flag-mA, Flag-mB, or Flag-mC. Cells were harvested and analyzed by flow cytometry. The full-length and mutant proteins were detected by Western blotting and were expressed to a similar extent after transfection. For each construct, the experiments were repeated at least twice. (D) U2OS cells were transiently transfected with Flag or Flag-SCAPER. They were either left untreated or treated with HU to enrich for G1/S cells. The percentage of mitotic cells in each population was determined by examining cells exhibiting condensed chromosomes marked with p-Ser10-H3. At least 100 cells were scored for each condition, and the experiments were repeated twice.

may occur at this stage in mitosis (unpublished data). Studies using deletion mutants showed that Flag-SCAPER-N, but not Flag-SCAPER-C, produced a similar cell cycle phenotype (Fig. 6 C), and a striking accumulation was also evident when cells were transfected with mutants mA and mC (Fig. 6 C). In contrast, expression of mutant mB, which lacks the ability to bind cyclin A/Cdk2, had only a modest effect on cell cycle progression (Fig. 6 C). Collectively, our data suggest that expression of SCAPER can affect cell cycle progression specifically in M phase by binding and sequestering cyclin A/Cdk2.

Depletion of SCAPER results in a delayed S phase reentry from quiescence

We investigated the effects of suppressing SCAPER production by RNAi-mediated down-regulation of endogenous SCAPER. siRNAs corresponding to four distinct SCAPER coding sequences were chosen to target SCAPER in human cells. Western blot analysis showed that treatment of cells with SCAPER siRNA resulted

in a substantial reduction (∼75%) in SCAPER expression (Fig. 7 C). Fluorescence-activated cell sorting (FACS) analysis of transfected cells indicated that cell cycle progression was not affected in cycling cells, and SCAPER siRNA-treated cells released from an HU block progressed through S phase and G2/M phase with kinetics similar to control cells (unpublished data). Microscopic inspection of transfected cells also did not reveal obvious abnormalities. In particular, immunofluorescence studies failed to detect any changes in the levels or in the subcellular localization of cyclin A, Cdk2, γ-tubulin, or calnexin. DNA staining also appeared similar in control and knock-down samples.

SCAPER expression is not cell cycle dependent (Fig. 2, D and E), and depletion of SCAPER had no obvious impact on cycling cells. However, the presence of abundant SCAPER in G0 cells (Fig. 2 D) prompted us to investigate the effect of SCAPER depletion on the ability of cells to exit the cell cycle or to reenter the cell cycle from quiescence (Fig. 7 A). First, there was no apparent impact of SCAPER depletion on entry into

A

Transfect cells with Control or SCAPER siRNA

↓

Arrest in G0

↓ ↘

Re-stimulate   Re-stimulate and HU block, 24 hours

↓   ↓

Analysis: Analysis:
FACS   FACS
Western
BrdU
Ki67
Subcellular Fractionation

C

Hours	0		12		15		18		21		24	
siRNA	Control	SCAPER-1	Control	SCAPER-1	Control	SCAPER-1	Control	SCAPER-1	Control	SCAPER-1	Control	SCAPER-1

SCAPER — 160 kD
β-tubulin — 55 kD

B

Hours	siRNA Control			siRNA SCAPER-1		
0	G0/G1	85%		G0/G1	90%	
	S	7%		S	3%	
	G2/M	8%		G2/M	7%	
12	G0/G1	85%		G0/G1	88%	
	S	4%		S	2%	
	G2/M	11%		G2/M	9%	
15	G0/G1	79%		G0/G1	92%	
	S	8%		S	3%	
	G2/M	13%		G2/M	5%	
18	G0/G1	58%		G0/G1	81%	
	S	40%		S	10%	
	G2/M	2%		G2/M	9%	
21	G0/G1	0%		G0/G1	42%	
	S	99%		S	57%	
	G2/M	1%		G2/M	1%	
24	G0/G1	2%		G0/G1	0%	
	S	38%		S	98%	
	G2/M	60%		G2/M	2%	

D   BrdU Positive Cells

Hours	Control siRNA	SCAPER-1 siRNA
0	8%	6%
12	15%	9%
14.5	39%	27%
16	71%	47%
17.5	80%	60%
19	86%	88%

E   Ki67 Positive Cells

Hours	Control siRNA	SCAPER-1 siRNA
0	32±3%	34±4%
3	43±2%	40±4%
6	50±4%	51±3%
9	75±5%	72±5%
21	100±0%	99±1%

F

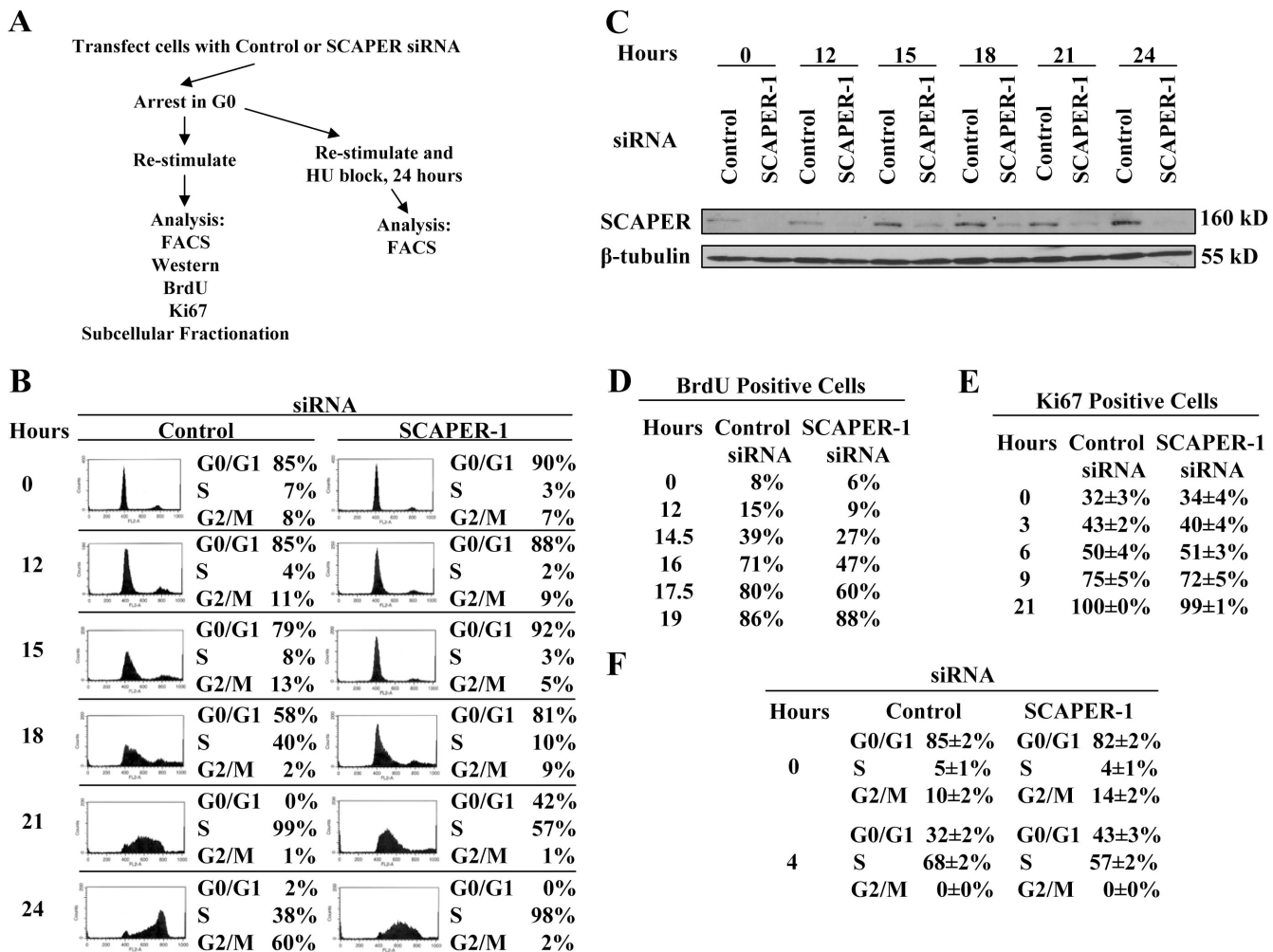| Hours	siRNA Control		siRNA SCAPER-1	
0	G0/G1	85±2%	G0/G1	82±2%
	S	5±1%	S	4±1%
	G2/M	10±2%	G2/M	14±2%
4	G0/G1	32±2%	G0/G1	43±3%
	S	68±2%	S	57±2%
	G2/M	0±0%	G2/M	0±0%

Figure 7.   **Ablation of SCAPER with siRNA results in a delayed G1/S transition upon cell cycle reentry from G0.** (A) An illustration of the experimental protocol for studying the effects of SCAPER depletion. T98G cells were transfected with either control or SCAPER siRNAs and were synchronized by serum deprivation. Cells were harvested at various time points upon restimulation. (B) FACS analysis. (C) Western blotting of endogenous SCAPER. β-Tubulin was used as a loading control. (D) Control or SCAPER-siRNA treated quiescent T98G cells were restimulated, and BrdU was added to the media 1 h before fixation. Cells were then stained with anti-BrdU antibody and DAPI. The percentage of BrdU-positive cells is presented. (E) Control or SCAPER-siRNA treated quiescent T98G cells were restimulated, fixed, and stained with anti-Ki67 antibody and DAPI. The percentage of Ki67-positive cells is presented. At least 100 cells were scored for each time point, and the experiments were performed twice. (F) Control or SCAPER-siRNA treated quiescent T98G cells were restimulated and blocked in G1/S with HU. They were subsequently released to allow entry into S phase.

quiescence (Fig. 7 B; row 1, columns 1 and 2), suggesting that ablation of SCAPER does not affect cell cycle exit in response to mitogen deprivation. Next, SCAPER-depleted cells brought to quiescence through serum deprivation were restimulated. As expected, a substantial number of cells transfected with control siRNA entered S phase ∼18 h after serum stimulation (40%, Fig. 7 B; row 4, column 1), and by 21 h, 99% of cells were in S phase (Fig. 7 B; row 5, column 1). 60% of cells progressed into G2/M phase 3 h later (Fig. 7 B; row 6, column 1). In striking contrast, the majority of SCAPER siRNA-treated cells were still in G1 18 h after stimulation (81%, Fig. 7 B; row 4, column 2). By 21 h after stimulation, a substantial proportion of these cells began to enter the S phase (57%) (Fig. 7 B; row 5, column 2). Only 2% of cells had progressed into G2/M phase after 24 h (Fig. 7 B; row 6, column 2). Thus, there appears to be a considerable delay (∼3 h in duration) in S phase entry associated with SCAPER depletion. Consistent with our FACS analysis, we observed

a comparable delay in BrdU incorporation associated with SCAPER depletion in siRNA-treated cells (Fig. 7 D). Importantly, this cell cycle delay phenotype was also observed with three additional, distinct siRNAs targeting SCAPER (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200701166/DC1). Furthermore, similar findings were observed in normal human cell lines such as IMR90 and WI38 (unpublished data), and in mouse 3T3 cells using a mouse-specific SCAPER siRNA (Fig. S1 B). Collectively, we conclude that SCAPER plays a critical role in promoting progression from G1 to S phase after cell cycle reentry from a quiescent state.

We attempted to pinpoint more precisely when the cell cycle delay takes place. We determined whether escape from the quiescence state was impaired in cells depleted of SCAPER by counting cells that expressed the Ki67 proliferation marker, apparent only in nonquiescent cells. Interestingly, both control and SCAPER siRNA-transfected cells were able to exit G0 with
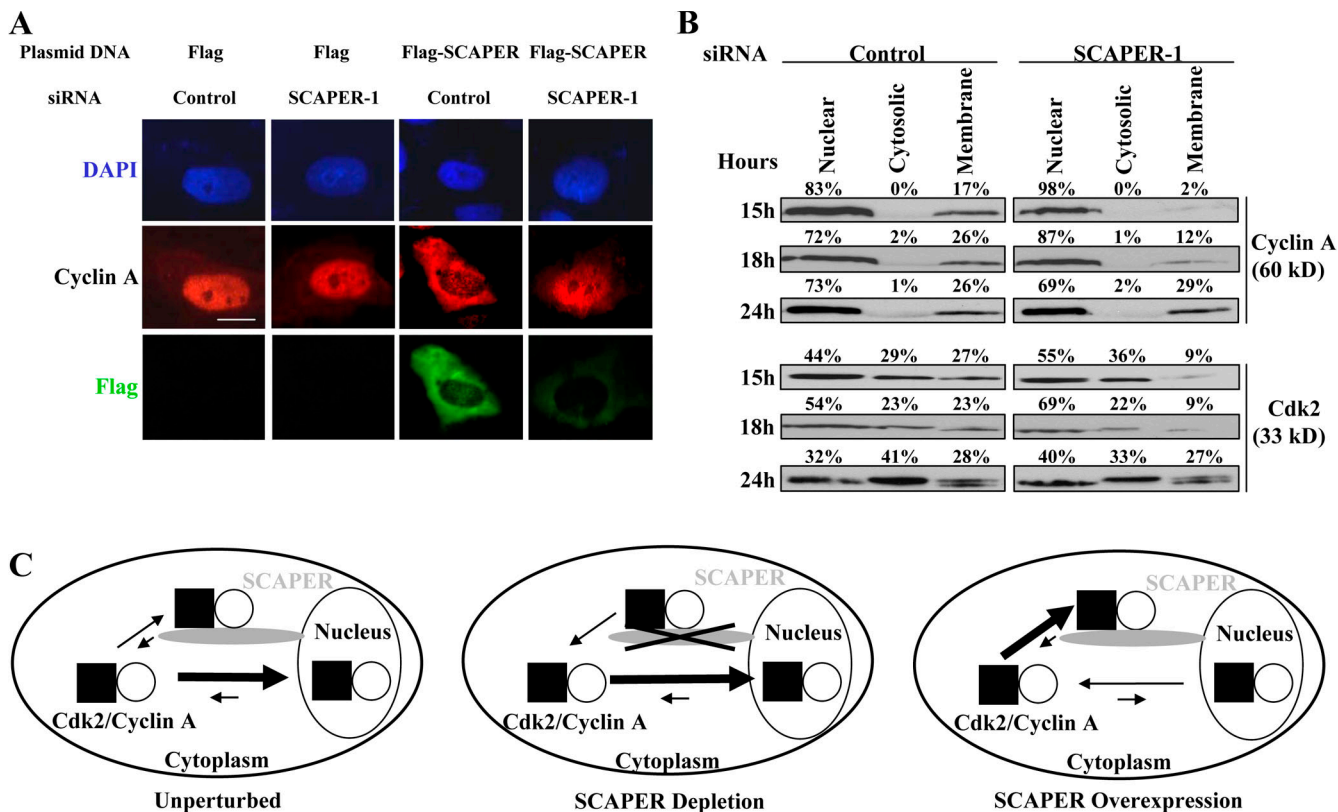
Figure 8.   **Ablation of SCAPER with siRNA releases cyclin A from the cytoplasm.** (A) U2OS cells were transfected with either Flag or Flag-SCAPER. 1 d later, they were transfected with either control or SCAPER siRNA. 1 d after siRNA transfection, cells were blocked with and released from HU, and processed for immunofluorescence studies with anti-Flag, anti-cyclin A antibodies, and DAPI. Bar, 10 μm. (B) T98G cells were transfected with either control or SCAPER siRNA and were synchronized in G0. Upon restimulation, cells were harvested and were subsequently subjected to subcellular fractionation. 50 μg of protein from nuclear, cytosolic, or membrane fraction were resolved by SDS-PAGE and blotted with the antibodies indicated. The percentage of cyclin A or Cdk2 in each fraction (expressed as a percentage of the total cellular cyclin A or Cdk2) is stated above each blot. (C) A model implicating SCAPER in the regulation of cyclin A/Cdk2 activity.

similar kinetics (Fig. 7 E). Furthermore, when G0 cells were released and synchronized at the G1/S boundary with HU, SCAPER depleted cells progressed into the S phase more slowly than control cells after release from the HU block (Fig. 7 F). Collectively, we believe that the S phase delay occurs in late G1/S and not earlier in the cell cycle (G0 or early G1 phase).

Ablation of SCAPER with siRNA alters cyclin A localization

We reported that overexpression of SCAPER results in the relocalization of cyclin A from the nucleus to the cytoplasm (Fig. 6 A). We sought to determine whether the converse is true, namely, whether cyclin A would relocalize from the cytoplasm when SCAPER function is suppressed. We and others have detected a portion of cyclin A residing within the cytoplasm and the microsomes (Girard et al., 1991; Carbonaro-Hall et al., 1993; Castro et al., 1994). This cyclin A partitioning is difficult to detect by immunofluorescence or live-cell imaging. Not surprisingly, when U2OS cells were transfected with either control or SCAPER siRNA, no difference in cyclin A staining in the cytoplasm was observed (Fig. 8 A, columns 1 and 2). To overcome this limitation, cells were first transfected with Flag-SCAPER to sequester cyclin A in the cytoplasm. They were subsequently treated with either control (Fig. 8 A, column 3) or SCAPER siRNA

(Fig. 8 A, column 4) to gauge the effects of knock-down. Upon silencing SCAPER, we observed recycling of cyclin A from the cytoplasm back to the nucleus (Fig. 8 A, column 4). Thus, SCAPER can influence the balance between cytoplasmic and nuclear pools of cyclin A, and it may represent a novel cyclin A/CDK2 regulatory protein that transiently maintains this kinase outside of the nucleus.

As a further confirmation that RNAi-mediated depletion of SCAPER alters cyclin A localization, we performed a series of subcellular fractionation experiments to examine the dynamic equilibrium of cyclin A in distinct cellular compartments, at a time when the cell cycle delay phenotype was first evident at late G1/S (15 and 18 h after serum stimulation as in Fig. 7 B, rows 3 and 4). For control siRNA-treated cells, we found that 17 and 26% of total cellular cyclin A was associated with the membrane compartment 15 and 18 h after stimulation, respectively (Fig. 8 B). Remarkably, the membrane-bound cytoplasmic pool of cyclin A was dramatically reduced when SCAPER was depleted. Thus, after SCAPER suppression, we observed only 2% of total cellular cyclin A associated with the membrane fraction 15 h after stimulation, and at 18 h after release, the percentage of cyclin A residing in the membrane increased to only 12% (Fig. 8 B). Consistent with decreased cyclin A retention at membranes, we observed a comparable reduction in

membrane-associated Cdk2 in SCAPER-depleted cells at 15 and 18 h after stimulation (Fig. 8 B). As a further test of the notion that partitioning of cyclin A within the cytoplasm by SCAPER occurred at a critical time in late G1/S phase when cyclin A levels were limiting, we compared the proportions of cyclin A in different compartments at a time when SCAPER is not thought to play a role, namely, after S phase entry (24 h, Fig. 8 B), and we did not observe any substantial difference between the two populations. In addition, consistent with cyclin A partitioning in SCAPER-depleted cells, no apparent difference in Cdk2 localization was observed in cells that had entered S phase (24 h, Fig. 8 B). In conclusion, our studies strongly support the notion that ablation of SCAPER decreases the pool of cyclin A in the cytoplasm and results in a delayed S phase reentry from quiescence. We have therefore for the first time unraveled the physiological significance of SCAPER and its role in maintaining cyclin A homeostasis in the cytoplasm.

Discussion

One enduring, fundamental issue in the field of cell cycle control pertains to our incomplete knowledge of Cdk substrates (Sanchez and Dynlacht, 2005). We isolated and purified a novel protein, SCAPER, which specifically interacts with cyclin A/Cdk2 both in vitro and in vivo. SCAPER is expressed in quiescent cells and its levels remain relatively unchanged throughout the cell cycle, suggesting that it may have a housekeeping function. It localizes primarily to the ER and interacts with cyclin A at multiple phases of the cell cycle. Progression through two key cell cycle transitions, G1/S and M phase, is differentially affected by SCAPER. Ablation of SCAPER by RNAi leads to delay of cell cycle reentry into S phase, but overexpression provokes cell cycle delay in M phase. These data lead us to propose an intriguing hypothesis: cytoplasmic cyclin A may be a limiting factor for the G1/S transition upon cell cycle reentry from quiescence; that is, cyclin A could be tethered to the ER, retaining it in the cytoplasm, where it may be required to phosphorylate a key substrate before S phase reentry. On the other hand, nuclear cyclin A may play a more prominent role in progression through the G2/M phase transition.

The presence of cyclin A/Cdk2 complexes in the cytoplasm is in accordance with the notion that cyclin A/Cdk2 phosphorylates substrates in that compartment as well as the nucleus. A requirement for cytoplasmic pools of cyclin A/Cdk2 is substantiated by recent studies implicating this kinase in mammalian centrosome duplication (Meraldi et al., 1999) and by other experiments that have identified Cdk2 targets associated with this organelle (Okuda et al., 2000; Fisk and Winey, 2001; Chen et al., 2002). Recent studies have shown that the nuclear import of cyclin A is much more rapid than its export (Jackman et al., 2002). In light of these observations, we believe that our identification of SCAPER has several important implications for the partitioning and regulation of cyclin A/Cdk2 activity and its targets (Fig. 8 C). First, because nuclear import of cyclin A/Cdk2 is kinetically favored over its export, it may be necessary to sequester the small quantities of this kinase that enter the cytoplasm at any given time. SCAPER, or an analogous cyclin A/Cdk2

binding protein, may perform such a function by partitioning cyclin A in the cytoplasm. In this regard, SCAPER may play a more prominent role during the G1/S transition in cells reentering the cell cycle from quiescence, whereas an analogous cyclin A/Cdk2 binding protein could substitute for SCAPER function at other stages of the cell cycle. Furthermore, we note that SCAPER is present in quiescent and early G1 phase cells. It could therefore play a second, "buffering" role, to scavenge small quantities of cyclin A present in early G1 cells that could trigger premature initiation of DNA replication. In contrast, cyclin A levels rise dramatically during S phase and could thereby exceed the amount of SCAPER, allowing nuclear localization of cyclin A/Cdk2. SCAPER could thus provide a threshold function, delicately balancing the amount of cyclin A/Cdk2 partitioned between the two cellular compartments (Fig. 8 C).

Although we do not yet know the precise mechanism by which SCAPER overexpression affects M phase, it does not appear to result from the direct attenuation of cyclin A/Cdk2 activity because excessive amounts of recombinant SCAPER do not substantially inhibit Cdk2 activity (Fig. 4 D and unpublished data). It is more likely that ectopic expression of SCAPER induces a cell cycle delay by sequestering the bulk of cyclin A/Cdk2 away from the pool of cyclin A complexes that normally interact with the Kip/Cip family of inhibitors (p21, p27) and the pRB family (p107 and p130) in the nucleus, thereby preventing the phosphorylation of critical substrates necessary for progression through M phase. Indeed, SCAPER represents a unique tool for dissecting the function of cyclin/Cdk complexes. It appears to be the first protein shown to preferentially interact with cyclin A/Cdk2 to the apparent exclusion of all other cyclin/Cdk complexes.

Recent evidence has implicated a role for cytoplasmic cyclin A in oncogenesis. Cytoplasmic accumulation of cyclin A has been reported in tumor cells (Aaltomaa et al., 1999a,b). In addition, targeting of cyclin A to the ER in normal rat fibroblasts leads to oncogenic activation and results in polyploidy, abnormal centrosome duplication, and genomic instability (Berasain et al., 1998; Faivre et al., 2002). These studies raise the intriguing possibility that an increase in cytoplasmic cyclin A levels is the direct cause of cellular transformation. As SCAPER possesses the ability to bind cyclin A in the cytoplasm, experiments are currently underway to determine precisely whether SCAPER up-regulation, which could lead to an increase of cytoplasmic cyclin A, is a common hallmark in cancer cells. If so, suppression of SCAPER function in tumor cells could be a major potential therapeutic application for SCAPER.

Materials and methods

Cell culture
HeLa, 293T, T98G, U2OS, IMR90, WI38, Saos2, and mouse C2C12 and 3T3 cells were grown in DME supplemented with 10% FBS at 37°C in a humidified 5% CO_2 atmosphere.

Production of recombinant proteins
Recombinant GST protein was expressed in bacteria transformed with pGEX2TK. GST-cyclin A/Cdk2 and GST-cyclin E/Cdk2 were produced in Hi5 insect cells by co-infection with viruses carrying GST-cyclin A and Cdk2 or GST-cyclin E and Cdk2. Fusion proteins were purified by affinity chromatography with glutathione agarose (Sigma-Aldrich) as described previously (Dynlacht et al., 1994). In brief, cells were lysed in 0.1 HEMGN

buffer (25 mM Hepes, pH 7.6, 100 mM KCl, 0.2 mM EDTA, pH 8.0, 12.5 mM MgCl$_2$, 0.1% NP-40, and 1 mM DTT with a cocktail of protease and phosphatase inhibitors). Cell extracts were incubated with glutathione agarose for 1 h at 4°C. The beads were collected by centrifugation, washed 4 times with 0.1 HEMGN buffer, and eluted with 20 mM glutathione in buffer containing 100 mM Tris (pH 7.9) and 120 mM NaCl. Flag-SCAPER-N and Flag-SCAPER-C were expressed in *Escherichia coli* strain BL21 transformed with RSET vectors containing Flag-SCAPER-N and Flag-SCAPER-C, and Flag-SCAPER was expressed in human 293 cells transfected with CBF-SCAPER, and purified with anti-Flag agarose (M2, Sigma-Aldrich). The relative protein concentrations were estimated by silver or Coomassie staining.

SCAPER purification and sequencing
Human 293 cells were lysed in ELB buffer (50 mM Hepes, pH 7.0, 250 mM NaCl, 0.5 mM EDTA, pH 8.0, 0.1% NP-40, and 1 mM DTT with a cocktail of protease and phosphatase inhibitors) for 30 min on ice and then centrifuged at 20,000 *g* for 30 min. The supernatant (1 g of total protein) was precleared three times by incubation with glutathione agarose loaded with ~50 μg of GST for 2 h at 4°C. The precleared supernatant was incubated with glutathione agarose loaded with ~40 μg GST-cyclin A/Cdk2 for 4 h at 4°C. The beads were collected by centrifugation and washed four times with ELB buffer. Finally, the beads were boiled in SDS sample loading buffer and analyzed by 10% SDS-PAGE. The gel was stained with silver. Immunodepletion and immunoprecipitation coupled to Western blotting with antibodies against p21, p27, p107, and p130 (Santa Cruz Biotechnology, Inc.) were used to identify each of the proteins in the eluate. The 158/160-kD band was excised from the gel, subjected to tryptic digestion, and the resulting peptides were separated by reverse-phase HPLC and analyzed with an LCQ quadrupole ion trap mass spectrometer (Finnigan) in the Harvard Microchemistry Facility.

SCAPER cloning and plasmid construction
SCAPER cDNA was generated using SuperScript II H⁻ Reverse Transcriptase (Life Technologies) according to the manufacturer's protocol. The primers for the reverse transcription reactions were AAC AAG GGT ACT CAA ATA CAG and AGC TCT TTC CCG GGC TGC ATC. The entire coding region was amplified by two PCR steps with PfuTurbo DNA polymerase (Stratagene) using the two fragments of the first strand of SCAPER cDNA as templates. The product was sub-cloned into an expression vector (CBF; M. Cole, Princeton University, Princeton, NJ) in frame with the N-terminal Flag tag to produce CBF-SCAPER.

CBF-SCAPER was digested with XmaI to produce two fragments of 2.2 kb and 6.7 kb, and the 6.7-kb fragment was sub-cloned into CBF XmaI site to generate CBF-SCAPER-N, which expresses amino acids 1–717 of SCAPER. The 6.7-kb fragment of DNA was treated with T4 polymerase to create blunt ends and re-ligated to generate CBF-SCAPER-C, which encodes amino acid residues 717–1399. The bacterial expression plasmids corresponding to SCAPER-N and SCAPER-C have an N-terminal Flag tag and were constructed by cloning each into the RSET vector (Invitrogen).

To identify potential cyclin binding motifs in SCAPER, the first three potential RXL sequences were changed to AAA to produce mutants mA, mB, and mC. Primers used in PCR mutagenesis were: AGC TAT TGC AGC AGC TGC TGT ACG ACC CTC CTC TGC and ACA GCA GCT GCT GCA ATA GCT TGG AGT GTT CCA CTA for mA, AAA ATT TGC AGC AGC TCG AGC ATT TGA TGT TAC ATT and GCT CGA GCT GCT GCA AAT TTT GGA GGT TCA ACT GGC for mB, TGC CTC TGC AGC AGC CTT GCG TTC CTG CAC AGC TTC and CGC AAG GCT GCT GCA GAG GCA GAG CGG CAG GCC CGT for mC. All constructs were verified by sequencing.

RNA interference
Synthetic siRNA oligonucleotides were obtained from Dharmacon. Transfection of siRNAs using Oligofectamine (Invitrogen) was performed according to the manufacturer's instructions. The 21-nucleotide siRNA sequence for the nonspecific control was 5′-AATTCTCCGAACGTGTCACGT-3′. The 21-nucleotide siRNA sequence for SCAPER-1 was 5′-GAATAAACGTCATGATGTTTT-3′.

RNA preparation and quantitative RT-PCR
Total RNA was prepared using Trizol Reagent (Life Technologies) according to the manufacturer's protocol. Semi-quantitative RT-PCR was performed using RT-PCR Superscript One Step kit (Invitrogen) as described by the manufacturer. Linear amplification was ensured in all cases. Primers for analysis of SCAPER expression were: CAG TGA TTT TTC TGC CAG CA and AGC TCT TTC CCG GGC TGC ATC; primers for analysis of β-actin levels were: ATC CTC ACC CTG AAG TAC CCC A and CTC GGC CGT GGT GGT GAA GCT GTA GCC GCG CT.

Cell cycle synchronization and FACS analysis
T98G cells were rendered quiescent by serum deprivation for 72 h and stimulated with 20% FBS to allow cell cycle reentry. Cell cycle synchronization of T98G and U2OS cells was also performed by drug treatment. G1, G1/S, and G2/M cells were obtained by treating the cells for 24 h with 0.4 mM mimosine, 2 mM hydroxyurea, and 40 ng/ml nocodazole, respectively. S phase cells were obtained by releasing cells from hydroxyurea treatment into fresh medium for 4–6 h. Cell cycle distribution was monitored by propidium iodide staining and FACS analysis was performed as reported previously (Woo et al., 1997).

Transient transfection, cell cycle analysis, and colony growth assay
Transient transfections were performed using calcium phosphate (Chen and Okayama, 1987). For cell cycle analysis, the indicated plasmids (20 μg) were cotransfected with pCMV-CD20 (2 μg) into human cells and processed as described previously (Woo et al., 1997). Cells were harvested with phosphate-buffered saline (PBS) containing 0.1% EDTA and stained with anti-CD20-FITC (Sigma-Aldrich) before FACS analysis.

Antibodies
To generate anti-SCAPER antibodies, a GST fusion protein encoding residues 382–493 was produced in bacteria and used to immunize rabbits after coupling to keyhole limpet hemocyanin. Polyclonal antibodies against SCAPER were purified using a GST-SCAPER fusion protein affinity column after the serum was precleared with a GST protein affinity column.

Immunoprecipitation, immunoblotting, and kinase assays
Cultured cells were washed once with PBS and lysed in ELB buffer. In a typical immunoprecipitation, whole-cell extract was incubated with antibodies for 2 h, and beads were washed four times with ELB buffer. Proteins bound to beads were eluted by boiling in sample buffer, separated by SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to Western blot analysis. All kinase reactions were performed at 30°C in buffer containing 25 mM Hepes, pH 7.6, 150 mM NaCl, 0.5 mM EDTA, pH 8.0, 10 mM MgCl$_2$, 1 μM ATP, 1 mM DTT and 5 μCi 32γ-ATP (NEN Life Science Products). For immunoblotting, antibodies against cyclin A, cyclin B, cyclin E, Cdk2, Cdk1, Sp1 (each from Santa Cruz Biotechnology, Inc.), calnexin (BD Biosciences), giantin (Covance Research Products, Inc.), actin, β-tubulin, and Flag (each from Sigma-Aldrich) were used.

Immunofluorescence
Indirect immunofluorescence was performed as described previously (Chen et al., 2002). In brief, cells were grown on glass coverslips, fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in PBS for 10 min followed by incubation in −20°C methanol for 2–3 min. The cells were permeabilized with 1% Triton X-100/PBS for 10 min. Slides were blocked with 3% BSA in 0.1% Triton X-100/PBS before incubation with primary antibodies. Antibodies against SCAPER, Flag, cyclin A, p-ser10-H3 (Upstate Biotechnology), BrdU (Sigma-Aldrich), and Ki67 (Zymed Laboratories) were used. Secondary antibodies used were Cy3- or FITC-conjugated donkey anti–mouse or anti–rabbit IgG (Jackson ImmunoResearch Laboratories). Cells were then stained with DAPI and slides were mounted, observed, and photographed using a Nikon Eclipse E800 microscope (63× or 100×, NA 1.4) equipped with a Photometrics Coolsnap HQ CCD camera. Images were acquired with MetaMorph7 (Molecular Devices).

Subcellular fractionation
Cells were incubated in hypotonic lysis buffer (10 mM Tris, pH 8.0, 10 mM NaCl, 3 mM MgCl$_2$, and 1 mM EGTA with a cocktail of protease and phosphatase inhibitors) for 10 min, followed by 20 strokes of a Dounce B homogenizer. The lysates were spun down for 5 min at 500 *g*, and the supernatant was designated the cytosolic fraction. The nuclear pellet was washed three times in wash buffer (hypotonic lysis buffer with 0.1% NP-40) and then treated with nuclear lysis buffer (20 mM Hepes, pH 8.0, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, and 0.5 mM DTT with a cocktail of protease and phosphatase inhibitors) for 30 min. The sample was spun at 14,000 *g* for 30 min, and the supernatant designated the nuclear fraction. The cytosolic fraction was spun at 100,000 *g* for 2 h, and the pellet (which was designated as the membrane fraction) was resuspended in ELB buffer with 1% NP-40. To verify the integrity of each fraction, Western blots were probed with nuclear (Sp1), cytosolic (β-tubulin), and membrane (calnexin and giantin) markers. For quantitation, 50 μg of protein from nuclear, cytosolic, or membrane fraction were resolved by SDS-PAGE and blotted with the antibodies indicated. The films were scanned with an Epson Perfection 4990 scanner and band intensities were

quantitated and analyzed using Bio-Rad Quantity One 1-D analysis software. The entire analysis was done within the linear range of detection.

Online supplemental material

Fig. S1 shows ablation of SCAPER with siRNA results in a delayed G1/S transition upon cell cycle reentry from G0. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200701166/DC1.

References

Aaltomaa, S., M. Eskelinen, and P. Lipponen. 1999a. Expression of cyclin A and D proteins in prostate cancer and their relation to clinopathological variables and patient survival. *Prostate.* 38:175–182.

Aaltomaa, S., P. Lipponen, M. Ala-Opas, M. Eskelinen, K. Syrjanen, and V.M. Kosma. 1999b. Expression of cyclins A and D and p21(waf1/cip1) proteins in renal cell cancer and their relation to clinicopathological variables and patient survival. *Br. J. Cancer.* 80:2001–2007.

Adams, P.D., W.R. Sellers, S.K. Sharma, A.D. Wu, C.M. Nalin, and W.G. Kaelin Jr. 1996. Identification of a cyclin-cdk2 recognition motif present in substrates and p21-like cyclin-dependent kinase inhibitors. *Mol. Cell. Biol.* 16:6623–6633.

Berasain, C., D. Patil, E. Perara, S.M. Huang, H. Mouly, and C. Brechot. 1998. Oncogenic activation of a human cyclin A2 targeted to the endoplasmic reticulum upon hepatitis B virus genome insertion. *Oncogene.* 16:1277–1288.

Bresnahan, W.A., I. Boldogh, T. Ma, T. Albrecht, and E.A. Thompson. 1996. Cyclin E/Cdk2 activity is controlled by different mechanisms in the G0 and G1 phases of the cell cycle. *Cell Growth Differ.* 7:1283–1290.

Carbonaro-Hall, D., R. Williams, L. Wu, D. Warburton, M. Zeichner-David, M. MacDougall, V. Tolo, and F. Hall. 1993. G1 expression and multistage dynamics of cyclin A in human osteosarcoma cells. *Oncogene.* 8:1649–1659.

Cardoso, M.C., H. Leonhardt, and B. Nadal-Ginard. 1993. Reversal of terminal differentiation and control of DNA replication: cyclin A and Cdk2 specifically localize at subnuclear sites of DNA replication. *Cell.* 74:979–992.

Castro, A., M. Jaumot, M. Verges, N. Agell, and O. Bachs. 1994. Microsomal localization of cyclin A and cdk2 in proliferating rat liver cells. *Biochem. Biophys. Res. Commun.* 201:1072–1078.

Chen, C., and H. Okayama. 1987. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* 7:2745–2752.

Chen, J., P. Saha, S. Kornbluth, B.D. Dynlacht, and A. Dutta. 1996. Cyclin-binding motifs are essential for the function of p21CIP1. *Mol. Cell. Biol.* 16:4673–4682.

Chen, Z., V.B. Indjeian, M. McManus, L. Wang, and B.D. Dynlacht. 2002. CP110, a cell cycle-dependent CDK substrate, regulates centrosome duplication in human cells. *Dev. Cell.* 3:339–350.

Coverley, D., H. Laman, and R.A. Laskey. 2002. Distinct roles for cyclins E and A during DNA replication complex assembly and activation. *Nat. Cell Biol.* 4:523–528.

den Elzen, N., and J. Pines. 2001. Cyclin A is destroyed in prometaphase and can delay chromosome alignment and anaphase. *J. Cell Biol.* 153:121–136.

Dynlacht, B.D., O. Flores, J.A. Lees, and E. Harlow. 1994. Differential regulation of E2F transactivation by cyclin/cdk2 complexes. *Genes Dev.* 8:1772–1786.

Faivre, J., M. Frank-Vaillant, R. Poulhe, H. Mouly, C. Jessus, C. Brechot, and J. Sobczak-Thepot. 2002. Centrosome overduplication, increased ploidy and transformation in cells expressing endoplasmic reticulum-associated cyclin A2. *Oncogene.* 21:1493–1500.

Fisk, H.A., and M. Winey. 2001. The mouse Mps1p-like kinase regulates centrosome duplication. *Cell.* 106:95–104.

Furuno, N., N. den Elzen, and J. Pines. 1999. Human cyclin A is required for mitosis until mid prophase. *J. Cell Biol.* 147:295–306.

Geley, S., E. Kramer, C. Gieffers, J. Gannon, J.M. Peters, and T. Hunt. 2001. Anaphase-promoting complex/cyclosome-dependent proteolysis of human cyclin A starts at the beginning of mitosis and is not subject to the spindle assembly checkpoint. *J. Cell Biol.* 153:137–148.

Girard, F., U. Strausfeld, A. Fernandez, and N.J. Lamb. 1991. Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. *Cell.* 67:1169–1179.

Hendzel, M.J., Y. Wei, M.A. Mancini, A. Van Hooser, T. Ranalli, B.R. Brinkley, D.P. Bazett-Jones, and C.D. Allis. 1997. Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma.* 106:348–360.

Howe, J.A., M. Howell, T. Hunt, and J.W. Newport. 1995. Identification of a developmental timer regulating the stability of embryonic cyclin A and a new somatic A-type cyclin at gastrulation. *Genes Dev.* 9:1164–1176.

Jackman, M., Y. Kubota, N. den Elzen, A. Hagting, and J. Pines. 2002. Cyclin A- and cyclin E-Cdk complexes shuttle between the nucleus and the cytoplasm. *Mol. Biol. Cell.* 13:1030–1045.

Klopfenstein, D.R., J. Klumperman, A. Lustig, R.A. Kammerer, V. Oorschot, and H.P. Hauri. 2001. Subdomain-specific localization of CLIMP-63 (p63) in the endoplasmic reticulum is mediated by its luminal alpha-helical segment. *J. Cell Biol.* 153:1287–1300.

Maridor, G., P. Gallant, R. Golsteyn, and E.A. Nigg. 1993. Nuclear localization of vertebrate cyclin A correlates with its ability to form complexes with cdk catalytic subunits. *J. Cell Sci.* 106:535–544.

Meraldi, P., J. Lukas, A.M. Fry, J. Bartek, and E.A. Nigg. 1999. Centrosome duplication in mammalian somatic cells requires E2F and Cdk2-cyclin A. *Nat. Cell Biol.* 1:88–93.

Murphy, M. 1999. Delayed early embryonic lethality following disruption of the murine cyclin A2 gene. *Nat. Genet.* 23:481.

Okuda, M., H.F. Horn, P. Tarapore, Y. Tokuyama, A.G. Smulian, P.K. Chan, E.S. Knudsen, I.A. Hofmann, J.D. Snyder, K.E. Bove, and K. Fukasawa. 2000. Nucleophosmin/B23 is a target of CDK2/cyclin E in centrosome duplication. *Cell.* 103:127–140.

Pagano, M., R. Pepperkok, F. Verde, W. Ansorge, and G. Draetta. 1992. Cyclin A is required at two points in the human cell cycle. *EMBO J.* 11:961–971.

Petersen, B.O., J. Lukas, C.S. Sorensen, J. Bartek, and K. Helin. 1999. Phosphorylation of mammalian CDC6 by cyclin A/CDK2 regulates its subcellular localization. *EMBO J.* 18:396–410.

Pines, J., and T. Hunter. 1991. Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. *J. Cell Biol.* 115:1–17.

Resnitzky, D., L. Hengst, and S.I. Reed. 1995. Cyclin A-associated kinase activity is rate limiting for entrance into S phase and is negatively regulated in G1 by p27Kip1. *Mol. Cell. Biol.* 15:4347–4352.

Romanowski, P., J. Marr, M.A. Madine, A. Rowles, J.J. Blow, J. Gautier, and R.A. Laskey. 2000. Interaction of Xenopus Cdc2 x cyclin A1 with the origin recognition complex. *J. Biol. Chem.* 275:4239–4243.

Saha, P., J. Chen, K.C. Thome, S.J. Lawlis, Z.H. Hou, M. Hendricks, J.D. Parvin, and A. Dutta. 1998. Human CDC6/Cdc18 associates with Orc1 and cyclin-cdk and is selectively eliminated from the nucleus at the onset of S phase. *Mol. Cell. Biol.* 18:2758–2767.

Sanchez, I., and B.D. Dynlacht. 2005. New insights into cyclins, CDKs, and cell cycle control. *Semin. Cell Dev. Biol.* 16:311–321.

Sherr, C.J., and J.M. Roberts. 2004. Living with or without cyclins and cyclin-dependent kinases. *Genes Dev.* 18:2699–2711.

Sweeney, C., M. Murphy, M. Kubelka, S.E. Ravnik, C.F. Hawkins, D.J. Wolgemuth, and M. Carrington. 1996. A distinct cyclin A is expressed in germ cells in the mouse. *Development.* 122:53–64.

Woo, M.S., I. Sanchez, and B.D. Dynlacht. 1997. p130 and p107 use a conserved domain to inhibit cellular cyclin-dependent kinase activity. *Mol. Cell. Biol.* 17:3566–3579.

Zhu, L., E. Harlow, and B.D. Dynlacht. 1995. p107 uses a p21CIP1-related domain to bind cyclin/cdk2 and regulate interactions with E2F. *Genes Dev.* 9:1740–1752.