Cell Cycle-dependent Subcellular Translocation of the Human DNA Licensing Inhibitor Geminin*

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Significance:

Multiple overlapping mechanisms are used by human cells to ensure genome stability.

Once per cell cycle replication is crucial for maintaining genome integrity. Geminin interacts with the licensing factor Cdt1 to prevent untimely replication and is controlled by APC/C-dependent cell cycle specific proteolysis during mitosis and in G1. We show here that human geminin, when expressed in human cells in culture under a constitutive promoter, is required for both nuclear exclusion and nuclear accumulation. Cdt1 overexpression targets geminin to the nucleus, while reducing Cdt1 levels by RNAi leads to the nuclear accumulation. Cdt1 overexpression targets geminin to the nucleus, while reducing Cdt1 levels by RNAi leads to the nuclear accumulation. Cdt1 overexpression targets geminin to the nucleus, while reducing Cdt1 levels by RNAi leads to the nuclear accumulation. Cdt1 overexpression targets geminin to the nucleus, while reducing Cdt1 levels by RNAi leads to the nuclear accumulation.

Geminin exclusion from the nucleus provides an additional level of licensing control, balancing the Cdt1/geminin ratio.

Conclusion:

Geminin exclusion from the nucleus provides an additional level of licensing control, balancing the Cdt1/geminin ratio.

Background:

The licensing inhibitor geminin must be regulated to ensure once per cell cycle replication.

Results:

Geminin is spatially and temporally controlled by nuclear exclusion during part of the G1 phase in human cells.

Discussion:

DNA Licensing Inhibitor Geminin*

Once per cell cycle replication is crucial for maintaining genome integrity. Geminin interacts with the licensing factor Cdt1 to prevent untimely replication and is controlled by APC/C-dependent cell cycle specific proteolysis during mitosis and in G1. We show here that human geminin, when expressed in human cells in culture under a constitutive promoter, is excluded from the nucleus during part of the G1 phase and at the transition from G0 to G1. The N-terminal 30 amino acids of geminin, which contain its destruction box, are essential for nuclear exclusion. In addition, 30 amino acids within the central domain of geminin are required for both nuclear exclusion and nuclear accumulation. Cdt1 overexpression targets geminin to the nucleus, while reducing Cdt1 levels by RNAi leads to the appearance of endogenous geminin in the cytoplasm. Our data propose a novel means of regulating the balance of Cdt1/geminin in human cells, at the level of the subcellular localization of geminin.

Before S phase onset, eukaryotic DNA is licensed for replication. Accurate regulation of replication licensing is necessary to maintain genome stability. Licensing, a highly conserved process from yeast to humans, involves the sequential recruitment and binding onto chromatin of the licensing factors ORC, Cdc6, Cdt1, and Mcm2-7, which form the prereplicative complex (1). Cdt1, an essential prereplicative complex component, is necessary for Mcm2-7 loading on chromatin (2, 3). Studies over the past years suggest that in metazoa, regulation of Cdt1 is critical for maintaining genome integrity (2, 4, 5).

A metazoan-specific inhibitor of licensing called geminin (6) binds and inhibits Cdt1, thereby preventing the recruitment of MCM proteins (7, 8). DNA replication licensing is therefore impeded during S, G2, and M phases of the cell cycle, where geminin is present (7, 8). Because depletion of geminin in Drosophila (9), human cells (10, 11), and early mouse embryos (12) leads to over-replication of the genome, it is believed that geminin is essential for genomic stability by preventing re-replication in the S-G2 phases in multicellular eukaryotes.

Cdt1/geminin balance at the different cell cycle stages is crucial for the maintenance of genome integrity (13). Geminin is proteolyzed by the anaphase-promoting complex/cyclosome (APC/C) at the metaphase to anaphase transition (6). However, in Xenopus egg extracts, part of the endogenous geminin escapes degradation in anaphase (14). This surviving population of geminin does not associate with Cdt1 and does not inhibit licensing (14), and this requires APC/C dependent ubiquitination of geminin (15, 16). These data suggest that upon exit from metaphase, geminin is altered so that its ability to inhibit Cdt1 is suppressed (14, 16). Geminin is reactivated as a Cdt1 inhibitor following import into the nucleus (14).

Studies in Xenopus egg extracts suggest that nuclear import of endogenous geminin not only re-enables it to bind Cdt1 and inhibit further origin licensing (14), but is also important to prevent re-replication during G2 (17). Cell cycle specific nuclear-cytoplasmic shuttling was reported as a regulatory mechanism for Xenopus and avian geminin (18, 19). However, in human cells, endogenous geminin appears exclusively nuclear and is only detectable during S and G2 phases (20–22).

Ectopic expression of a non-degradable form of geminin during G1 has been reported to diminish loading of the MCM complex on chromatin, thus inhibiting DNA replication (23) and eliciting apoptosis in various cancer cell lines but not in primary fibroblasts (24). Furthermore, siRNA suppression of geminin activity leads to proliferation arrest only in cancer-derived cells (25). Geminin or agents mimicking its action have therefore been proposed as promising candidates for anti-tumor drug development (24), further emphasizing the need for an accurate understanding of the regulation of geminin within human cells.

Accurate regulation of licensing during the different aspects of the life of metazoan cells is ensured by multiple, overlapping mechanisms, which include nuclear exclusion, Cdt1/geminin balance, and nuclear import/export. The elucidation of these regulatory mechanisms is essential to understand the role of geminin in maintaining genome stability.
Geminin Nuclear Exclusion during \( G_1 \)

Timely licensing is particularly important when quiescent cells enter the cell cycle. Several prereplicative complex components, such as Cdc6, Cdt1, and Orc1 as well as the licensing inhibitor geminin are E2F targets (23, 27–29). Mechanisms in addition to transcriptional regulation must therefore ensure that a window of opportunity is opened for licensing when the cells enter the cell cycle. Cdc6 phosphorylation by cyclin E and its resulting protection from APC/C-mediated proteolysis has previously been shown to contribute to this control (30). Here, we show that an additional level of control operates in human cells. Expression of geminin through a constitutive promoter in human cells results in its nuclear exclusion during part of the \( G_1 \) phase and at the transition from quiescence to proliferation. Nuclear exclusion requires the amino-terminal 30 amino acids of geminin, which include its destruction box. Co-expression of Cdt1 targets geminin to the nucleus. We suggest that regulation of geminin through changes in its subcellular localization provides a fail-safe mechanism for ensuring a tight balance of Cdt1 and geminin in the nucleus, thereby controlling timely licensing.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfections, and Synchronization**—MCF7 cells were grown in standard DMEM medium with 10% FBS. Transfections were carried out with FuGENE (Roche Diagnostics). Cells were analyzed 22 h post transfection. Stable cell lines were selected on 500 \( \mu \text{g/ml} \) G418. Synchronizations were performed by incubation with nocodazole (50 ng/ml) for 16 h, followed by mitotic shake-off and replating of mitotic cells. Withdrawal of MCF7 cells to \( G_0 \) was achieved by incubation with 5 \( \mu \text{M} \) tamoxifen for 48 h.

**siRNA for Cdt1**—The siRNA sequence targeted to Cdt1 is as follows: 5’- CAUGAUACACUUUGGCCUU-3’ (Dharmacon). As control, siRNA against luciferase (known as GL2-Dharmacon) was used. 0.8 \( \times \) 10\(^5\) MCF7 cells were plated in a 35-mm dish. Cells were transfected twice with a 24-h interval at 150 \( \mu \text{M} \) using DharmafECT and analyzed 72 h after the first transfection.

**Plasmid Construction**—hGem cDNA was amplified by PCR for the introduction of restriction sites and was cloned either as a HindIII/KpnI fragment amino-terminally of GFP (Gem-GFP) or as EcoRI/Xhol fragment carboxyl-terminally of GFP (GFP-Gem) into vector pcDNA3.1-EGFP. Constructs expressing the amino-terminal part of geminin (amino acids 1–79 of human geminin, Gem(1–79)), or the carboxyl-terminal part of geminin (Gem(76–209)) were created by PCR and subcloned into the pcDNA3.1-EGFP vector amino-terminally of GFP, between the KpnI and BamHI sites of Gem-GFP. Cdt1-dhcRed fusion was constructed by subcloning of full-length Cdt1 (2) into pdiHCRed-N1 (kindly provided by J. Ellenberg) between the Nhel and KpnI sites (33), Cdt1Δ170–190, kindly provided by Dr. Helin (31), was subcloned into pcDNA3.1/EGFP (Invitrogen) as described previously (33). For Gem-NLS-GFP construction, three copies of the SV40 nuclear localization sequence and a single Myc epitope were inserted between the KpnI and BamHI sites of Gem-GFP. Cdt1-dhcRed fusion was constructed by subcloning of full-length Cdt1 (2) into pdiHCRed-N1 (kindly provided by J. Ellenberg) between the Nhel and KpnI sites (33), Cdt1Δ170–190, kindly provided by Dr. Helin (31), was subcloned into pcDNA3.1/EGFP (Invitrogen) as described previously (33).

**Immunofluorescence and Microscopy**—Immunofluorescence was performed as described previously (21) after fixation of cells with 4% paraformaldehyde. Primary antibodies used were against cyclin A (Neomarkers, 6E6, 1:80), geminin (1:2000) (20), GFP (1:1000), MCM2 (BD Biosciences, 1:500), whereas secondary antibodies were from Molecular Probes (Alexa Fluor 488 and 568). To analyze chromatin binding of MCM2, the immunofluorescence protocol was followed (21) with the following modifications: cells were washed once with CSK buffer (10 mm PIPES, 300 mM sucrose, 100 mM NaCl, and 3 mM MgCl\(_2\)) and incubated with CSK + 0.5% Triton X-100 twice for 1 min each before fixation. Before blocking, cells were washed three times with PBS, once with 0.5% Nonidet P-40 in PBS and three times with PBS. Following mounting with Vectashield containing DAPI, samples were observed with a Nikon Eclipse TE2000-U microscope, and images were taken by a Nikon Digital Sight DS-L1 camera. For Fig. 4, cells were stained with Draq5 and visualized with a Leica SP5 Confocal Microscope. When percentages of cells are shown, >200 cells were measured in each case.

**RESULTS**

**hGem Is Specifically Excluded from the Nucleus when Ectopically Expressed During the \( G_1 \) Phase**—Endogenous human geminin localizes to the nucleus, is detected exclusively during \( S \) and \( G_2 \) phases of the cell cycle (21, 22), and is regulated both transcriptionally (23) and by APC/C-mediated proteolysis during \( G_1 \) (6). Ectopic expression of geminin in \( G_1 \) has been shown to lead cancer cells, but not normal cells, into apoptosis (24).

To study the subcellular behavior of hGem in human cancer cells, amino- and carboxyl- terminal fusions of geminin with green fluorescent protein (GFP-Gem and Gem-GFP, respectively) driven by the CMV promoter were constructed, and their localization was examined following transient transfection in the human breast cancer cell line MCF7. Although GFP-fused forms of geminin localize to the nucleus in a subpopulation of cells, a large fraction (approximately half) of transfected cells show exclusion from the nuclear compartment (Fig. 1A and quantification in Fig. 1B). Nuclear exclusion of human geminin is not due to the presence of a tag on the protein, as it is also observed by immunofluorescence using an untagged geminin expression construct (Fig. 1C, upper panels) and is not specific for MCF7 cells, as it is also observed in HeLa cells (Fig. 1C, lower panels) and HEK293 cells (data not shown). This is consistent with the results of Boos et al. (18) who reported that following transfection in human cells, human geminin is less able to localize to the nucleus than *Xenopus* geminin.

To examine whether nuclear exclusion of geminin is cell cycle phase-dependent, double immunofluorescence was carried out using cyclin A as a molecular indicator of the \( S \) and \( G_2 \) phases of the cell cycle (26). Timely licensing is particularly important when quiescent cells enter the cell cycle. Several prereplicative complex components, such as Cdc6, Cdt1, and Orc1 as well as the licensing inhibitor geminin are E2F targets (23, 27–29). Mechanisms in addition to transcriptional regulation must therefore ensure that a window of opportunity is opened for licensing when the cells enter the cell cycle. Cdc6 phosphorylation by cyclin E and its resulting protection from APC/C-mediated proteolysis has previously been shown to contribute to this control (30). Here, we show that an additional level of control operates in human cells. Expression of geminin through a constitutive promoter in human cells results in its nuclear exclusion during part of the \( G_1 \) phase and at the transition from quiescence to proliferation. Nuclear exclusion requires the amino-terminal 30 amino acids of geminin, which include its destruction box. Co-expression of Cdt1 targets geminin to the nucleus. We suggest that regulation of geminin through changes in its subcellular localization provides a fail-safe mechanism for ensuring a tight balance of Cdt1 and geminin in the nucleus, thereby controlling timely licensing.
phases of the cell cycle (34). As shown in Fig. 2, A and B, cells showing nuclear exclusion of Gem-GFP do not co-express cyclin A, suggesting that exogenous human geminin is excluded from the nucleus during part of the G₁ phase. In contrast, endogenous geminin is normally not expressed during G₁ (Fig. 2, A and C). Therefore, this suggests that ectopic expression of geminin during G₁ leads to its nuclear exclusion.

To investigate further the cell cycle-specific pattern of geminin localization, we constructed a cell line stably expressing a mutant form of geminin bearing a point mutation at its destruction box (Gem(L26A) (7)) tagged with GFP. This mutant shows the same subcellular localization as wild type geminin upon transient transfection (see below). Following mitotic arrest by nocodazole and mitotic shake off, cells were released from the block and collected after 2, 4, 10, 13, 16, and 24 h. Gem(L26A)-GFP is detected at low levels soon after mitotic exit and accumulates during G₁, showing that it is partially degraded at mitosis. This suggests that the point mutation introduced is not sufficient to block recognition by APC/C and degradation of geminin (data not shown). In early time points following a mitotic block, Gem(L26A)-GFP is present at low levels and appears in the nucleus (Fig. 3A). Gem(L26A)-GFP is detected in the cytoplasm from 10 h after release (Fig. 3A), whereas nuclear exclusion is observed 16 h after release, as cyclin A levels increase (Fig. 3B), marking entry into S phase. Nuclear exclusion is always observed in cyclin A-negative cells (data not shown), as also shown for the transiently transfected wild type protein above. Similar results were obtained using a cell line stably expressing wild type human geminin tagged with GFP (data not shown). Hence, we conclude that nuclear exclusion of Gem-GFP constitutively expressed in cycling human cells takes place at late time points upon release from mitosis into G₁.

Based on the observation that geminin nuclear exclusion appears in G₁ phase, we examined its behavior at the transition from quiescence to the cell cycle. During cell cycle reentry, licensing must take place before S phase onset. However both

FIGURE 1. Subcellular localization of GFP-fused forms of geminin. A, MCF7 cells were transfected with Gem-GFP and GFP-Gem constructs. DNA was stained with DAPI, and the localization pattern of the constructs was determined by GFP fluorescence. B, Quantification of nuclear (white), partly nuclear and partly cytoplasmic (Nuc+Cytopl; gray), and exclusively cytoplasmic (nuclear exclusion; black) phenotypes observed for Gem-GFP and GFP-Gem are shown as the percentage (%) of GFP-positive cells. 200 cells were counted. C, upper panels: MCF7 cells were transfected with plasmid expressing non-tagged geminin (Geminin-pcDEBD), and geminin localization was determined by indirect immunofluorescence with a specific anti-geminin antibody (upper right). Lower panels: HeLa cells were transfected with Gem-GFP, and the localization of GFP fluorescence was determined. Cells with transfected geminin excluded from the nucleus (stained with DAPI, left panels) are readily detected.

FIGURE 2. Cell cycle-dependent localization of Gem-GFP. A, asynchronous MCF7 cells transiently transfected with Gem-GFP (upper panels) and non-transfected MCF7 cells (lower panels) were analyzed by immunofluorescence using cyclin A as a marker of S and G₂ phases (red). GFP fluorescence (upper panels) and anti-geminin immunofluorescence (lower panels) are shown in green, and nuclear staining (DAPI) is shown in blue. B and C, quantification of the percentage (%) of Gem-GFP or geminin-positive cells, respectively, based on cyclin A status and subcellular localization. In contrast to endogenous geminin, which is mainly detected in the nucleus (white) in cyclin A-positive cells (S/G₂ phases), cells with nuclear exclusion of Gem-GFP (black) are always cyclin A-negative (G₁ cells). 200 cells were counted. Nuc+Cytopl, partly nuclear and partly cytoplasmic.
Geminin Nuclear Exclusion during G1

FIGURE 3. Study of Gem(L26A)-GFP localization in synchronized MCF7 cells. A, MCF7 cells were synchronized in mitosis by a nocodazole block and mitotic shake-off. After release and as cells progressively enter G1 and S/G2 phases, the subcellular localization of Gem(L26A)-GFP was analyzed at the indicated time points. Quantification of nuclear (white), partly nuclear and partly cytoplasmic (Nucl+Cytopl; gray), and exclusively cytoplasmic (nuclear exclusion, black) phenotypes observed for Gem(L26A)-GFP are shown as the percentage (%) of GFP-positive cells. B, indirect immunofluorescence for cyclin A was performed at the same time points to estimate when cells are in G1 phase (negative for cyclin A staining) and when they enter into S/G2 phases (positive for cyclin A staining). Gem(L26A)-GFP appears in the nucleus soon after the mitotic block when cells are negative for cyclin A staining. Detection of Gem(L26A)-GFP in the cytoplasm is observed 10 h after release, whereas the nuclear exclusion phenotype is detectable 16 h after release when cyclin A levels increase.

GFP is detected in the cytoplasm at early time points. Its nuclear exclusion reaches a peak at 6 h (Fig. 4B and data not shown), whereas its cytoplasmic localization decreases as Cdt1 levels increase. Indeed, concomitant localization of Gem-GFP and Cdt1 (Fig. 4A) shows that geminin is in the cytoplasm when Cdt1 levels are low in cells (0- to 9-h time points). As Cdt1 levels increase, geminin colocalizes with Cdt1 in the nucleus (arrows in Fig. 4A, 12- to 21-h time points) and then retains its nuclear localization following Cdt1 proteolysis after entry into S phase (24-h time point). Experiments with MCF7 cells stably expressing Gem(L26A)-GFP showed similar results (data not shown). Immunofluorescence of endogenous geminin in the same experimental setup in the parental MCF7 cell line (Fig. 4D) shows that endogenous geminin levels increase after the G1/S phase transition, and geminin is undetectable in the earlier time points, consistent with its transcriptional regulation by E2F and its APC/C-mediated proteolysis (35). Taken together, these data suggest that geminin ectopically expressed during the G0 to cell cycle transition is unable to reach the nucleus until Cdt1 levels increase. Our data suggest that an additional level of control exists in human cells, which balances levels of Cdt1 and geminin in the nucleus during the G1 phase.

Mutant Analysis Maps Regions Responsible for Nuclear Exclusion—To investigate which regions of human geminin are responsible for nuclear localization and nuclear exclusion, a series of geminin constructs fused to GFP were created (Fig. 5A), and their localization was tested following transient transfection. A diagrammatic representation of the known domains of hGem is shown in Fig. 5B. As shown in Fig. 5A (quantification of images) deletion of either the amino-terminal half (Gem(76–209)) or the carboxyl-terminal half (Gem(1–79)) of human geminin leads to abolishment of the exclusive cytoplasmic and the net nuclear phenotype, thus indicating that both regions of the molecule are required for accumulation of geminin in nucleus or cytoplasm. Weak nuclear activity, more evident in stable cell lines, was observed for Gem(76–209) (Fig. 5A and data not shown).

Human geminin has two clusters of basic amino acids, which could act as nuclear localization sequences: one close to the amino terminus (amino acids 50–54, KRKRE) and the second in the middle of the molecule (amino acids 105–108, KRRK). Previous work has implicated the first of these regions in the nuclear localization of Xenopus geminin (17, 36) and the second in the nuclear localization of human geminin (18). Close to the first cluster, a serine residue competent for phosphorylation (Ser-49) is conserved in mammals. We therefore deleted this conserved region from hGem (Gem∆NLS) to examine if this signal also serves as a NLS in humans. However, this deletion has no effect on the subcellular localization of geminin (Fig. 5, A and C), suggesting that this sequence does not serve as the main NLS sequence in human geminin. Similarly, mutating Ser-49 to alanine or glutamic acid does not affect the localization of geminin (data not shown). Gem(76–209), which contains the second basic amino acid cluster (107KRRK108) shows a weak NLS activity. Gem∆90–120, which lacks the second putative NLS sequence, as well as the interaction domain with Cdt1, has lost the exclusive cytoplasmic and net nuclear phenotype (Fig. 5, A–C), suggesting that this region of the molecule is necessary
for both nuclear accumulation and nuclear exclusion. Addition of NLS sequences from SV40 (Gem-NLS) leads to 100% accumulation of geminin in the nucleus. Close to the N terminus of geminin lies a destruction box (a consensus sequence of nine amino acids, \(23^{\text{RRTLKMIQP}}31\)). A mutation in this box (Gem(L26A)), which we showed to be insufficient to inhibit geminin destruction during mitosis, has no effect on the subcellular localization of transfected geminin. Deletion, however, of the first 30 amino acids of geminin (Gem/H9004\(^{1–30}\)), almost entirely abrogates nuclear exclusion of the molecule, suggesting that this region plays a central role in nuclear exclusion (Fig. 5C). On the contrary, Gem/Δ170–209, which lacks the interaction domain with the chromatin modifiers Brm1/Brm (37), phosphorylation sites by CKII (32, 38) and mimics a truncated molecule produced by caspase-3 cleavage (32), exhibits the same localization pattern as Gem-GFP. From this mutation analysis, we concluded that the amino-terminal 30 amino acids of the human geminin, which contain its destruction box, are required for specific exclusion from the cell nucleus. The central part of the molecule (amino acids 90–120), which mediates interactions of geminin with Cdt1, is required for both nuclear exclusion and nuclear accumulation.

FIGURE 4. Gem-GFP localization upon cell cycle reentry. MCF7 cells stably expressing Gem-GFP were synchronized in G0 phase by the addition of tamoxifen. Upon drug removal, cells were released from G0, and samples were collected at the indicated time points. A, Representative images from Gem-GFP expressing MCF7 cells after immunofluorescence for Cdt1 in asynchronous (untreated) and tamoxifen-treated cells. DNA was stained with Draq5 (blue), the localization pattern of Gem-GFP was determined by GFP fluorescence (green), and Cdt1 expression was determined by indirect immunofluorescence (red). + Tam, treated with tamoxifen cells. White arrows mark cells co-expressing Cdt1 and exogenous geminin. B, quantification of the subcellular localization of Gem-GFP expressing MCF7 cells upon release from G0 after tamoxifen treatment. Nuclear (white), partly nuclear and partly cytoplasmic (Nucl + Cytopl; gray), and exclusively cytoplasmic (Cytopl.; black) phenotypes are shown as the percentage (%) of GFP-positive cells. C, percentage (%) of cells positive for Cdt1, cyclin A, and BrdU in Gem-GFP MCF7 cells at indicated time points upon tamoxifen removal. D, percentage (%) of cells positive for Cdt1, chromatin-bound MCM2 (following pre-extraction), geminin, cyclin A, and BrdU in parental MCF7 cells at indicated time points upon tamoxifen removal. 400 cells were counted.
Cdt1 Targets Geminin to the Nucleus through Direct Interaction

Given the tight correlation we observed between geminin nuclear localization and Cdt1 levels upon entry to the cell cycle (Fig. 4A), we examined whether the interaction of geminin with its binding partner, Cdt1, could alter its subcellular distribution. For this purpose, we performed co-transfection...
FIGURE 6. Cdt1 targets Gem-GFP to the nucleus. A, MCF7 cells were transiently co-transfected with Gem-GFP and dhcRed (control) or with Cdt1dhcRed. Co-transfection of Cdt1dhcRed, but not of dhcRed, leads to nuclear localization of Gem-GFP. B, accumulation of geminin in the nucleus after co-transfection with Cdt1. First row, the localization of GFP-Gem is depicted in MCF7 cells. Cotransfection with Cdt1 leads to geminin accumulation in the nucleus (second row). A mutant form of Cdt1, Cdt1(161-end), which lacks the essential region for nuclear localization, fails to target geminin to the nucleus upon co-transfection (third row). However, addition of a nuclear localization sequence to Cdt1(161-end), creating Cdt1-NLS(161–end), restores the ability of Cdt1 to target geminin to the nucleus upon co-transfection (fourth row). C, co-transfection of Gem-GFP with Cdt1Δ170–190dhcRed, a mutant form that lacks the interaction domain for geminin, fails to target Gem-GFP to the nucleus compared with Cdt1dhcRed. D, a mutant form of geminin, GemΔ90–120-GFP, which lacks the interaction domain for Cdt1, has lost the ability to exclusively localize to the nucleus upon co-transfection with Cdt1dhcRed. Co-transfection of Gem-GFP with Cdt1dhcRed is shown for comparison. E, silencing of endogenous Cdt1 using siRNA affects nuclear accumulation of endogenous geminin. In Cdt1-depleted cells, distribution of endogenous geminin to both the nucleus and cytoplasm is observed. siRNA against luciferase (control siRNA) has no affect on the nuclear localization of geminin. 200 cells were counted. Nucl + Cytopl, partly nuclear and cytoplasmic.
Geminin Nuclear Exclusion during G1

whereas addition of nuclear localization sequences from SV40 to Cdt1(161-end) (Cdt1-NLS(161-end)) restores the ability of Cdt1 to target geminin to the nucleus (Fig. 6B). A mutant form of Cdt1 (Cdt1Δ170–190), which is deficient for interaction with geminin (31), fails to target geminin to the cell nucleus (Fig. 6C), whereas a mutant form of geminin (GemΔ90–120), which is deficient for interaction with Cdt1, is not targeted to the nucleus by Cdt1 co-expression (Fig. 6D). Taken together, these data suggest that Cdt1 can target geminin to the nucleus through direct interactions.

To investigate whether depletion of Cdt1 could affect the nuclear localization of endogenous geminin, siRNAs against Cdt1 were used, and the localization of endogenous geminin was assessed using specific antibodies. As shown in Fig. 6E opposite to untreated cells, Cdt1 silencing results in the appearance of geminin in the cytoplasmic compartment.

DISCUSSION

Here, we demonstrate subcellular changes of geminin as a novel mechanism of regulating the Cdt1/geminin ratio in human cells. Specifically, ectopically expressed hGem in a cancer-derived cell line, shows nuclear exclusion during part of the G1 phase. Domain mapping analysis identifies two regions responsible for nuclear exclusion: one in the amino-terminal and another in the central part of geminin. Co-transfection and siRNA experiments of Cdt1 demonstrate its importance for targeting geminin to the nucleus.

Cell Cycle-dependent Nucleocytoplasmic Shuttling of Geminin—The importance of geminin function for genomic stability has been extensively studied in multicellular organisms (9–12). Geminin binds and inhibits Cdt1, thus preventing re-replication in the S-G2 phases. For this function, the nucleocytoplasmic shuttling of geminin has been reported to play an important role in Xenopus and in avian systems (17, 19). Endogenous Xenopus geminin is capable of binding recombinant Cdt1 and inhibiting further origin licensing only after its nuclear import into an interphase nucleus (14), whereas NLS-deleted X. laevis geminin fails to inhibit re-replication induced by Cdt1 in Xenopus egg extracts (17). Avian geminin, which is present in the nucleus during S and G2 phases, inhibits the MCM chromatin loading and represses Hox gene expression, whereas in G1 phase, translocation of geminin to the cytoplasm ensures proper MCM loading onto chromatin and Hox gene transcription. In human cells, however, endogenous geminin appears to be exclusively present in the nucleus during S and G2 phases (21, 22), whereas it is not detectable in G1 phase due to APC/C-dependent proteolysis.

We show here that in human cancer cells, when geminin is expressed under a constitutive promoter it exhibits cell cycle dependent nucleocytoplasmic shuttling, which is correlated with cell cycle phase and Cdt1 expression. In cycling cells, geminin is excluded from the nucleus in cells which do not express cyclin A, and are therefore in G1. A fraction of cyclin A-negative cells, however, do localize geminin to the nucleus, suggesting that nuclear exclusion takes place during part of the G1 phase. Consistently, when cells are synchronized in mitosis and released into the G1 phase, geminin is present in the nucleus at early time points, shortly after mitotic release when its levels are low and is specifically excluded from the nucleus late in G1 phase, preceding the G1/S transition. Nuclear localization of geminin in G1 appears therefore correlated with Cdt1 expression, which accumulates in the nucleus in early G1, and its levels decrease close to the G1/S phase transition (21). Consistently, approximately half of the cells showing geminin exclusion in cycling cells are negative for Cdt1 (data not shown). In addition, geminin appears in the cytoplasm when quiescent human cancer cells reenter the cell cycle. Detailed time course analysis shows that this exclusion takes place when Cdt1 levels are low, in early time points upon cell cycle reentry. As Cdt1 levels increase, geminin co-localizes with Cdt1 in the nucleus. Earlier work has shown that a Cdt1-geminin complex is functional for licensing (39, 40) and that the stoichiometry of Cdt1 and geminin (39) as well as structural changes (40) dictate permissive versus inhibitory licensing activity of the complex. These data allow us to speculate that this control mechanism over geminin balances the levels of geminin in the nucleus to the levels of Cdt1, permitting licensing when cells transit from quiescence to G1 (Fig. 7). As cells proceed into S phase, this control over geminin localization is relieved, and excess geminin enters the nucleus to inhibit further licensing. Geminin exclusion from the nucleus may act synergistically to CDK-dependent Cdc6 stabilization in post-quiescent cells, thus providing a window of opportunity for the licensing process to take place.

Domains Responsible for the Nucleocytoplasmic Phenotype Observed in Human Geminin—The sequence of human geminin has two regions that could act as nuclear localization sequences: one close to the amino terminus (amino acids 50–54) that has been implicated in the nuclear localization of Xenopus geminin (17, 36) and another one in the middle of the molecule (amino acids 105–108) that has been implicated in the nuclear localization of human and avian geminin (18, 19). Detailed analysis of geminin mutant forms showed that deletion of the corresponding Xenopus NLS has no effect on the localization of human geminin, whereas deletion of the second region (amino acids 90–120), where also the interaction domain with Cdt1 lies, leads to abolishment of the exclusive cytoplasmic and net nuclear phenotype. This indicates that this region is responsible for nuclear accumulation and for nuclear exclusion. Consistently, weak NLS activity is attributed to region 76–209 of geminin.

In addition, the first 30 amino acids in the amino-terminal part of the molecule, where the destruction box is located, are required for its nuclear exclusion. Perhaps, the amino-terminal region of geminin, which is well conserved among species, contains signals responsible either for its export or its retention to the cytoplasm. In Xenopus and avian geminin, a Crm1-dependent export mechanism has been described (18, 19). However, in human cells, leptomycin B treatment did not reveal Crm1-dependent nuclear export of geminin (data not shown), leading us to favor a mechanism that involves retention of geminin to the cytoplasm.

Nuclear Import of Human Geminin through Direct Interaction with Cdt1—In addition to the above-mentioned sequence (KRKK) that could serve as nuclear localization signal, a Cdt1-dependent mechanism is also important for the nuclear
localization of geminin. Interestingly, these residues are part of the coil-coiled domain of geminin, which is responsible for the interaction with Cdt1. Co-transfection experiments of wild type and mutant forms in the interaction domain of Cdt1 and geminin suggest that Cdt1 mediates geminin nuclear accumulation through direct interaction. Likewise, reduction of Cdt1 endogenous levels by siRNA leads to the appearance of geminin in the cytoplasm. Our data point toward a model where Cdt1 in G1 binds geminin, thus forming a complex that enters the nucleus. We have also previously shown that Cdt1 recruits geminin onto chromatin in human cells (33). A balanced stoichiometry between these two molecules would be achieved by coupling geminin nuclear entry to the levels of Cdt1 during G1, ensuring fine regulation of both activities and ultimately tight control over DNA replication (Fig. 7). In mitosis, a different mechanism balancing Cdt1 to geminin levels by controlling Cdt1 proteolysis has been reported (31). We have also shown that Idas, another geminin partner can target geminin to the nucleus (41), which may provide an additional level of control.

As cells enter S phase, Cdt1 is proteolyzed in the nucleus, releasing geminin, whereas the mechanism retaining geminin in the cytoplasm appears to be inactive. As the region responsible for geminin nuclear exclusion overlaps with its destruction box, we favor the hypothesis that APC/C inactivation in S phase may be linked to loss of geminin nuclear exclusion. It is therefore clear that multiple overlapping pathways cooperate to tightly control Cdt1 to geminin stoichiometry within the human cell nucleus.

So far, no specific modification responsible for the observed cell cycle-dependent nucleocytoplasmic phenotype of geminin has been identified. However, we do not exclude that an alteration of geminin may influence its availability to its different interaction partners and its subcellular distribution.

Functional Importance of Geminin Nucleocytoplasmic Shuttling—Overexpression of geminin in mammalian cells has been shown to lead to cell-specific responses. Particularly, ectopic overexpression of geminin in various cancer cell lines during G1 has been shown to lead to irreversible S phase arrest and apoptosis, whereas primary cells arrest in G1 without showing apoptotic behavior (24). Therefore, gaining a better insight into the regulation of geminin in human cancer cells when it is ectopically overexpressed is of great importance. Our results show that in human cells geminin is regulated at the level of its subcellular localization. Although this mechanism seems to act redundantly in human cultured cells, as geminin is not detected in G1 or G0 phase due to E2F transcriptional regulation and APC/C-dependent proteolysis, this subcellular regulation of geminin may have a functional relevance in specific cell types or

FIGURE 7. Proposed model for the regulation of licensing through nuclear exclusion of geminin. Human cancer cells that enter the cell cycle from quiescence (G0 phase) regulate the licensing inhibitor geminin at the level of subcellular distribution. In particular, shortly after the transition from G0 to G1, geminin is excluded from the cell nucleus. As Cdt1 accumulates, it binds to geminin, and they enter the nucleus, probably as a complex. Degradation of Cdt1 following the G1/S transition leads to geminin excess in the nucleus and licensing inhibition. pre-RC, prereplicative complex. G, Geminin; ORC, origin recognition complex.
Geminin Nuclear Exclusion during G₁ under specific circumstances. Interestingly, geminin has been detected by immunohistochemistry in the cytoplasm of melanoma patient cells but shows a predominantly nuclear staining in uninvolved epidermal cells (42), indicating that the spatial regulation of geminin might be compromised in cancer cells. Further experiments are needed to elucidate in depth the complex regulatory mechanisms of geminin and their role in cancer biology.

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