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Regulation of Geminin Functions by Cell Cycle-Dependent Nuclear-Cytoplasmic Shuttling▽

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The geminin protein functions both as a DNA rereplication inhibitor through association with Cdt1 and as a repressor of *Hox* gene transcription through the polycomb pathway. Here, we report that the functions of avian geminin are coordinated with and regulated by cell cycle-dependent nuclear-cytoplasmic shuttling. In S phase, geminin enters nuclei and inhibits both loading of the minichromosome maintenance (MCM) complex onto chromatin and *Hox* gene transcription. At the end of mitosis, geminin is exported from nuclei by the exportin protein Crm1 and is unavailable in the nucleus during the next G_1 phase, thus ensuring proper chromatin loading of the MCM complex and *Hox* gene transcription. This mechanism for regulating the functions of geminin adds to distinct mechanisms, such as protein degradation and ubiquitination, applied in other vertebrates.

For DNA replication initiation during the cell cycle, the origin recognition complex binds to chromatin at the replication origins and recruits the initiation factors Cdc6 and Cdt1, which are in turn required for loading of the minichromosome maintenance (MCM) complex onto chromatin to form the prereplicative complex (2). After DNA replication is initiated, it is critical to ensure that the replication origins do not refire in the same cell cycle in order to maintain the genetic stability of an organism. As one of the redundant mechanisms for inhibiting rereplication, geminin protein accumulates in the nucleus in early S phase and binds to Cdt1, thus sequestrating Cdt1 from binding to the MCM complex as well as DNA. Consequently, the MCM complex cannot be reloaded onto the replicated chromatin and rereplication is inhibited (36, 38, 40). From the end of mitosis onward, the presence of geminin in the nucleus needs to be prevented in the next G_1 phase in order to enable Cdt1 to license the next round of DNA replication (24). In higher eukaryotes, geminin serves as a major DNA replication safeguard (27).

Although geminin is conserved in metazoans, distinct mechanisms are adopted to inactivate it at the end of mitosis. Geminin contains a destruction box sequence that mediates anaphase promoting complex (APC)-dependent ubiquitination and proteolysis (17, 25). In mammalian as well as *Drosophila* cells, geminin is inactivated by APC-dependent degradation at the end of mitosis and accumulates again in the nucleus in the next S phase (25, 31, 38). However, in *Xenopus* eggs, 30 to 60% of geminin protein resists degradation at the end of mitosis. Cyclin-dependent kinase activity and APC-dependent transient polyubiquitination without proteolysis are essential for geminin inactivation and DNA replication licensing (14, 21). Thus, mechanistic distinction and redundancy for regulating the function of geminin were suggested for metazoans.

The embryonic patterning control genes of the *Hox* family are activated in nonidentical, overlapping expression domains, in colinearity with their organization in genomic clusters (7, 18). Various qualitative and quantitative combinations of Hox proteins specify embryonic structures along the body axis during development (12, 16). In addition to the function of Cdt1 sequestration, geminin was recently reported to associate with the *Hox*-repressive polycomb complex as well as the *Hox*-regulatory DNA elements on chromatin, thus repressing *Hox* gene transcription (23). Furthermore, geminin antagonizes the functions of Hox and Six3 proteins through direct protein-protein interactions. The interaction of Hox or Six3 proteins with geminin is competitive to the interaction of geminin with Cdt1, allowing for a coordination between the cell cycle and embryonic patterning (6, 22, 23, 30).

Nuclear-cytoplasmic shuttling is one of several critical mechanisms for regulating the function of molecules in cellular processes. Among the transporter proteins, Crm1 functions as a Ran-binding nuclear transport receptor, which is in charge of the nuclear export of shuttling proteins with a consensus leucine-rich nuclear export signal (NES; LXXLXXLXL) (4, 11). The exportin function of Crm1 can be specifically blocked by the fungal toxin leptomycin B (LMB) through a covalent modification (9, 19, 28).

In this paper, we demonstrate that the avian homolog of geminin functions as a repressor of MCM loading and *Hox* gene transcription like geminin in mammals. Avian geminin contains a consensus NES sequence that does not exist in mammalian geminin. We suggest a novel regulatory mechanism of avian geminin by Crm1-dependent nuclear-cytoplasmic shuttling. This shuttling is coordinated with different cell cycle phases and regulates the availability of geminin in the

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nucleus as an inhibitor of both MCM loading and *Hox* gene transcription.

MATERIALS AND METHODS

Isolation of avian geminin. Total RNA was prepared (Micro RNA isolation kit; Stratagene) from anterior neural plates together with underlying layers dissected from chick embryos before overt neural folding (HH5). cDNA was generated using a SMART cDNA library construction kit (Clontech). Since the 5′ primer of the SMART kit contained an ATG codon which could interfere with the proper translational initiation of the transgene during the overexpression analysis, we used a modified SMART III primer (AAG CAG TGG TAT CAA CGC AGA GTG GCC ATT ACG GCC GGG). The cDNA was PCR amplified (18 cycles) and digested with the restriction enzyme SfiI, for which two different unique restriction sites were introduced into the cDNA at the 5′ and 3′ ends by the primers during the PCR (GGCCATTACGGCC and GGCCGCCTCGGCC, respectively). The cDNA was then purified from an agarose gel and cloned directly into an expression vector, followed by transformation into *Escherichia coli*. Colonies hybridizing to cDNA from HH3 neural plates were excluded from further analysis (90% of the clones). The DNA sequences of 400 clones were determined, and the expression patterns of novel, putative full-length cDNAs including geminin were analyzed in chick embryos.

Cell culture, treatments, immunocytochemistry, synchronization, and cell phase separation. Chick embryonic fibroblasts (CEFs) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal calf serum (FCS), 2% chicken serum, 2 mM glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, sodium pyruvate, 1,000 mg/liter glucose, and pyridoxine at 37°C under 5% CO_2. The cells were transfected using a calcium phosphate transfection kit (Invitrogen) as described by the manufacturer. Treatment with 20 ng/ml LMB (Sigma) was done for 2 h. Antibodies were raised against recombinant murine geminin and purified by affinity chromatography (23). The anti-geminin antibody detects a single band on Western blots of chick cell extracts (data not shown) that does not show after knockdown with specific small interfering RNA (siRNA) (see Fig. 4B). Cell fixation, permeabilization, and immunofluorescent staining using antibodies against geminin (1:1,000) were performed as described previously (32). Quantitation of immunofluorescence data was performed by counting at least 100 cells with specific subcellular localizations in different microscopic viewing areas.

Since the duplication time of CEFs is about 30 h, the majority of cells were synchronized to the G_1-S border and G_2/M phase after a 24-hour treatment with 5 μg/ml aphidicolin (Sigma) and 0.4 μg/ml nocodazole (Sigma), respectively. Cells were harvested by trypsinization. The S-phase cells were harvested after 3 h of release in fresh Dulbecco's modified Eagle's medium from a 24-hour aphidicolin treatment, while the G_1-phase cells were harvested after 3 h of culture from a collection of mitotic shake-off cells. The collected cells were washed with ice-cold phosphate-buffered saline (PBS) once and resuspended in 1 ml PBS. Then, the cells were pressed through a syringe into 3 ml 100% ethanol and incubated on ice for at least 30 min. The cells were subsequently spun down and dried before their resuspension in 425 μl PBS. Fifty microliters RNase and 25 μl propidium iodide were then added to the cell resuspension to final concentrations of 1 μg/ml and 10 μM, respectively. The samples were incubated at room temperature for 30 min in the dark, followed by flow cytometry analysis using a FACSCalibur flow cytometer (Becton-Dickinson Biosciences) and data analysis using ModFit LT 3.0 (Verify Software House Inc., ME).

For nuclear and cytoplasmic phase separation, the synchronized cells were incubated with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.2 mM EGTA, 20 mM NaF, 10% glycerol, and 0.5% NP-40 on ice for 5 min. The samples were spun down at 4°C for 15 min at full speed, and the supernatants were collected as cytoplasmic fractions. The pellets were washed with lysis buffer twice and sonicated in the lysis buffer to obtain the nuclear fractions. Western blot analyses were performed on each sample using antibodies against geminin (1:200) (23), α-tubulin (1:4,000; Sigma), or LaminB (1:1,000; Santa Cruz).

Chromatin isolation, limb bud mesenchymal culture, and reverse transcription (RT)-PCR. For chromatin isolation, the cultured CEFs were transfected with plasmids or siRNAs using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. Treatment with 10 ng/ml LMB was applied for 16 h to the culture cells or after siRNA transfection. Then, the chromatin phase of the cells was isolated as previously described (39) and loaded onto 10% sodium dodecyl sulfate-polyacrylamide gels. Western blot analyses were performed using antibodies against MCM3 (kind gift from D. Maiorano) or histone 2B (1:2,000; Upstate).

For the culture of primary mesenchymal cells, limb buds of stage HH19 embryos were isolated in calcium-magnesium-free saline glucose solution (CMFSG; 50 ml 1% glucose and 50 ml 10× CMFS including 0.185 g KCl, 0.015 g KH_2PO_4, 4 g NaCl, 0.114 g $NaHCO_3$, 0.063 g NaH_2PO_4-H_2O in a total volume of 500 ml solution). Then, the limb buds were digested in 4 ml digestion solution (10% chicken serum, 10% collagenase, 10 μl trypsin-EDTA in 10 ml CMFSG) for 50 min at 37°C. The digestion was stopped by the addition of an equal amount of 10% FCS. The cells were briefly vortexed to totally dissociate them and then passed through a cell strainer (Falcon) to avoid cell aggregates. The filtered cells were centrifuged at 230 × *g* for 10 min, and the pellet was resuspended in 2 ml Ham's F12 culture medium (Invitrogen) containing 10% FCS and 0.2% chicken embryo extracts. The cells were counted by a hematocytometer and distributed as 2×10^5 aliquots in four-well culture plates (Nunc). The cells were then incubated for 1 h at 37°C under 5% CO_2 so that the cells were attached to the plate. Then, the cultured limb bud mesenchymal cells were transfected using Lipofectamine 2000 (Invitrogen) as described by the manufacturer or treated with 10 ng/ml LMB. Twenty-four hours after transfection or LMB treatment, the total RNA of the cells was isolated using an RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. For the detection of *Hoxd13* transcription levels, RT-PCR for *Hoxd13* (5′-CTATAGCTGCAGGATGTCC-3′, 5′-CGATTTCCAGAAGTGCGAGC-3′) and the internal control *GAPDH* (5′-ACGCCATCACTATCTTCCAG-3′, 5′-CAGCCTTCACTACCCTCTTG-3′) was carried out in the same reaction mixture using a one-step RT-PCR kit (QIAGEN).

In ovo electroporation and whole-mount in situ hybridization. In ovo electroporation of stage HH9-11 chick embryos and analysis of *Hoxb9* transcription by whole-mount in situ hybridization were performed as described previously (23, 35).

RESULTS

Cell cycle- and Crm1-dependent nuclear-cytoplasmic shuttling of geminin. Chick geminin consists of 223 amino acids, with 47%, 50%, and 53% identity to the proteins in *Xenopus* spp., mouse, and human, respectively. There are two highly conserved domains with more than 80% identity, comprising a destruction box and a coiled coil domain (Fig. 1A). The latter, together with its N-terminally adjacent region, interacts with other proteins, including geminin itself, Cdt1, and homeodomain proteins (3, 20, 23, 29, 33, 37). Only chick and *Xenopus* geminin proteins possess consensus NES in the C-terminal region (Fig. 1A, green).

The subcellular localization of chick geminin was investigated by immunofluorescence staining of primary CEFs (Fig. 2A). In comparison with the subcellular localization of geminin in mammalian cells (23, 25), there are two remarkable differences. First, chick geminin was detected in all cultured CEFs, suggesting that geminin was present in every cell cycle phase. Second, geminin was found in both the cytoplasm and the nucleus in about 20 to 30% of the cells, whereas it localized exclusively in the cytoplasm in the other 70 to 80% (Fig. 2A). To further investigate whether the subcellular localization of geminin is cell cycle dependent, CEFs were synchronized by arresting cells at the G_1-S border, arresting and releasing them in the S phase, and arresting them at the G_2/M phase and characterized by flow cytometry (Fig. 2B). The three synchronized CEF populations contained 72%, close to 100%, and 71% of their cells at the G_1-S border, in the S phase, and in the G_2/M phase, respectively. They were fractionated into cytoplasmic and nuclear extracts, which were characterized by Western blotting using antibodies against α-tubulin as a cytoplasmic and LaminB as a nuclear marker (Fig. 2C). In S- and G_2/M-phase extracts, geminin was found mainly in the nuclear fraction, with a lower level also detectable in the cytoplasm. In extracts of cells at the G_1-S border, a phase in which replication origins may already begin to be fired, geminin was pre-

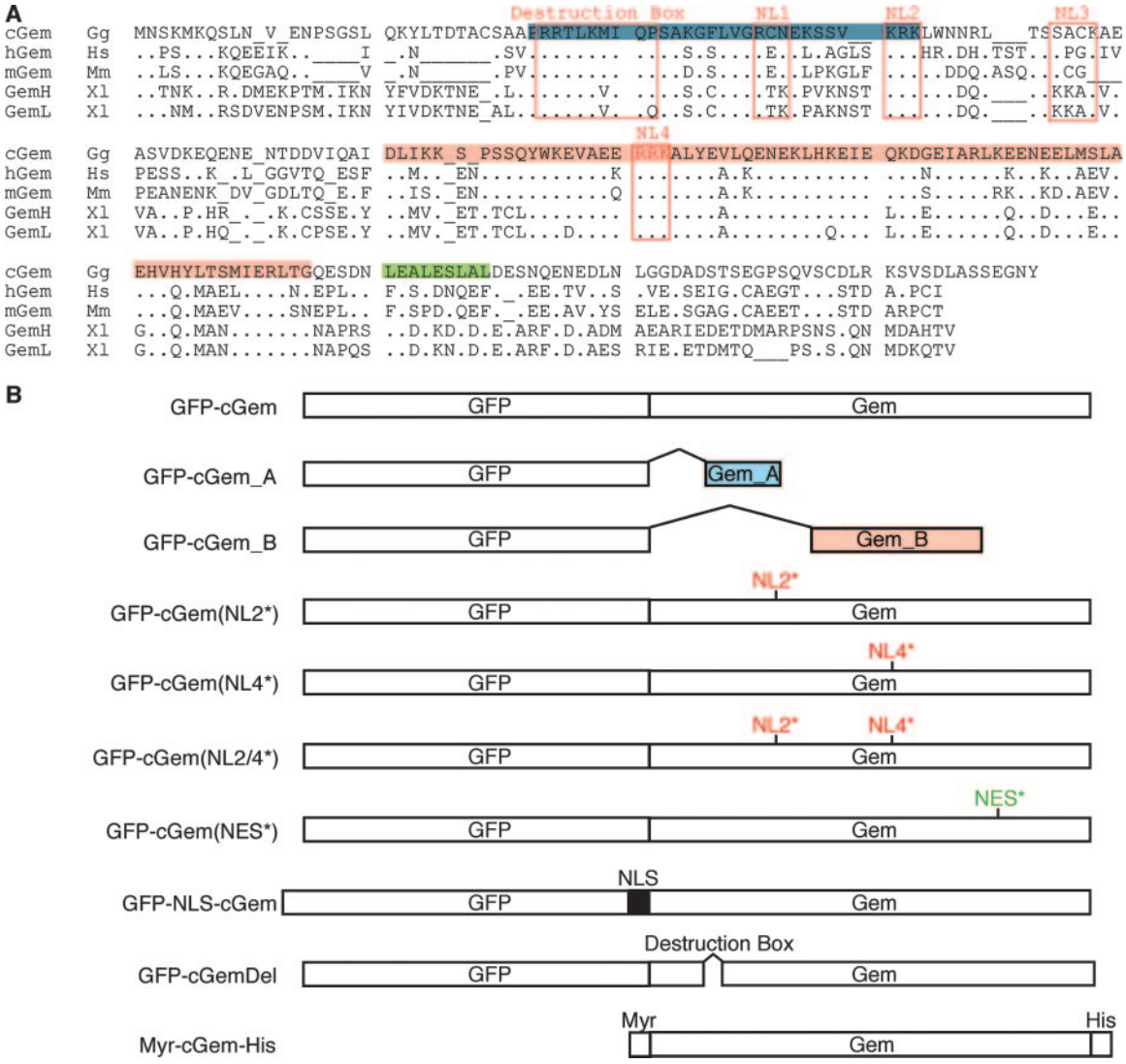

FIG. 1. Sequence of avian geminin and schematic representation of its modified forms. (A) Comparison of the predicted amino acid sequences of chick, human, mouse, and *Xenopus* geminin proteins. The cGem_A domain (amino acids 31 to 59) is highlighted in blue, cGem_B (amino acids 93 to 165) is highlighted in light red, the destruction box is indicated, the leucine-rich NES is shown in green, and the four basic amino acid motifs potentially involved in nuclear localization are boxed in red (NL1, NL2, NL3, and NL4). (B) Schematic representation of different modified forms of chicken geminin.

dominantly detected in the cytoplasm. In G_1-phase cells prepared by a short-term culture of mitotic shake-off cells, the subcellular localization of geminin was determined to be exclusively cytoplasmic by immunocytochemistry (Fig. 2D). This suggests that geminin shuttles into the nucleus in the S phase, maintains its nuclear presence during the S, G_2, and early M phases, and is exported before entering the next G_1 phase. The predominantly cytoplasmic geminin in asynchronously growing CEFs was shifted to the nucleus by exposure to LMB (Fig. 2A and E), indicating that the nuclear export of geminin was Crm1 dependent.

Nuclear localization of avian geminin. The functional determination of nuclear localization signals (NLS) in *Xenopus* geminin had revealed three basic amino acid clusters (NL1, NL2, and NL3) (Fig. 1A) that constitute two classical bipartite elements (NL1-NL2 and NL2-NL3) (3, 5). Murine and human geminin proteins lack a bipartite NLS. Human geminin requires within the coiled-coil domain a conserved cluster of the basic amino acids RRK (here defined as NL4) (Fig. 1A) and seems to employ Cdt1 for nuclear import (5, 43). In the chick geminin protein, the NL1 motif is only weakly conserved, while the third motif (NL3) is not conserved at all (Fig. 1A). To characterize the nuclear import signals of avian geminin, two green fluorescent protein (GFP)-fused, truncated forms of geminin were generated. One contained amino acids 31 to 59 (Fig. 1A, blue), thus including the destruction box and NL1 and NL2 but not the coiled-coil domain (Fig. 1B, GFP-cGem_A). The other contained amino acids 93 to 165 (Fig. 1A, light red), including NL4 but excluding the NES (Fig. 1B, GFP-cGem_B). Overexpression of these protein domains led to their enrichment in the nuclei of transfected cells (Fig. 3A and B), suggesting that the NL2 motif is sufficient to bring the

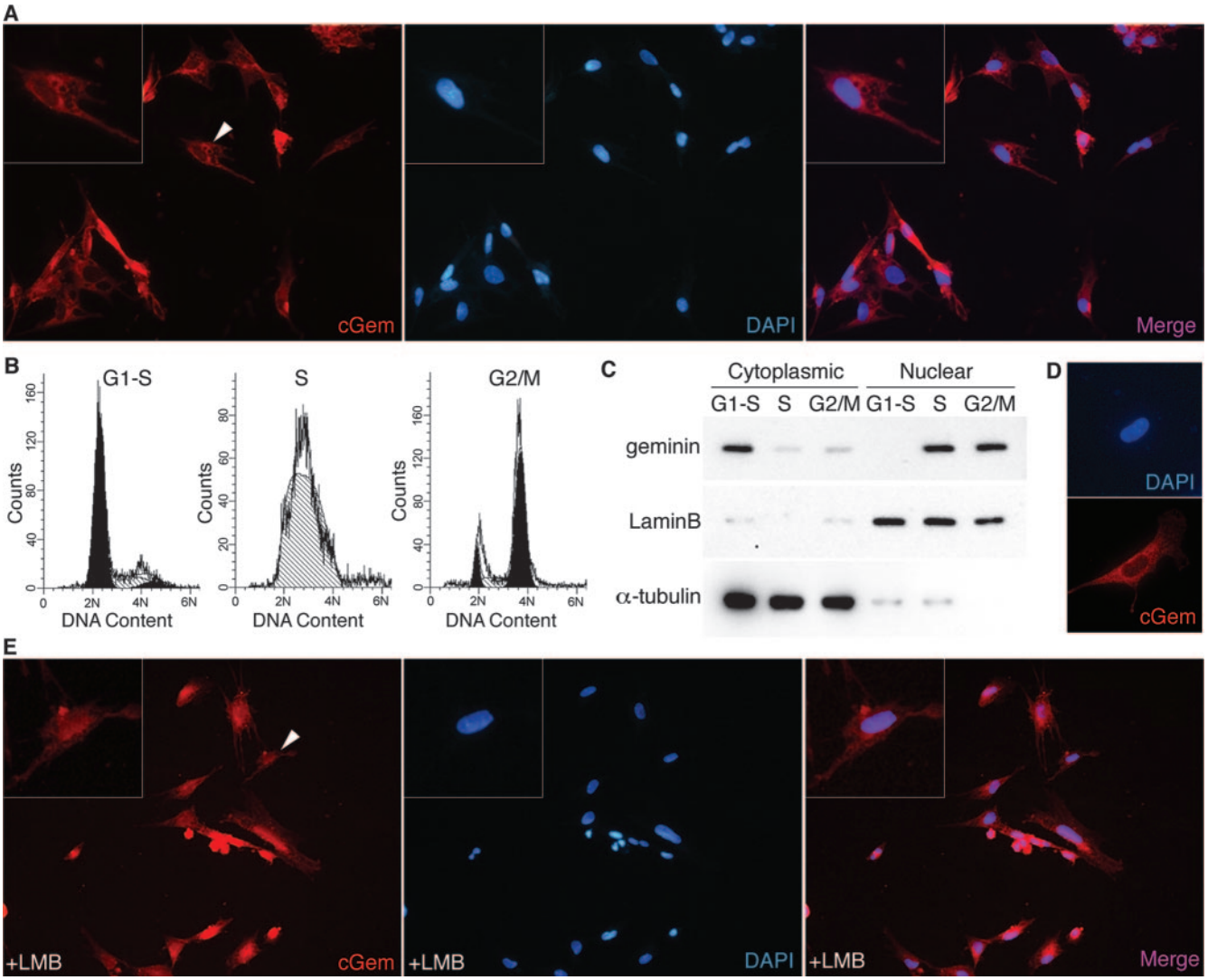

FIG. 2. Cell cycle- and Crm1-dependent subcellular localization of chicken geminin. (A) Immunostaining for endogenous geminin protein in unsynchronized CEFs with anti-geminin antibodies. Note that all the cells are stained with geminin antibodies, the majority displaying exclusively cytoplasmic staining. The arrowhead marks the cell enlarged in the upper-left corner. (B) CEF synchronization. Three synchronized CEF populations were obtained, with about 72% of the cells in the G_1-S border, close to 100% in the S phase, and 71% in the G_2/M phase. (C) The subcellular localization of geminin in extracts from cell populations enriched for different cell cycle phases. (D) Cytoplasmic localization of geminin in G_1-phase cells. (E) The nuclear enrichment of geminin after 2 h of LMB treatment. The arrowhead marks the cell enlarged in the upper-left corner. DAPI, 4′,6′-diamidino-2-phenylindole.

A domain into the nucleus, while the nuclear transportation of the B domain may involve NL4.

To determine the amino acids responsible for the nuclear import of avian geminin, three mutated forms of geminin were generated (Fig. 1B). In GFP-cGem(NL2*) and GFP-cGem(NL4*), basic amino acids in NL2 and NL4 were mutated to alanine, respectively, while both motifs were mutated in GFP-cGem(NL2/4*). After LMB treatment, the GFP-cGem(NL2*) and GFP-cGem(NL4*) proteins were enriched in the nucleus, whereas the GFP-cGem(NL2/4*) protein was predominantly localized in the cytoplasm (Fig. 3C to H). This result indicates that the NL2 motif in domain A and the NL4 motif in domain B play redundant roles as nuclear import signals. If both motifs are mutated, geminin loses its capability to shuttle into the nucleus (Fig. 3G and H). Modified forms of geminin were generated in order to further investigate the functional relevance of nuclear-cytoplasmic shuttling of avian geminin, and their subcellular localizations were examined. The GFP-cGem and cGem-His fusion proteins were located in the cytoplasms of more than 80% of the transfected cells (Fig. 3I and J), whereas GFP-cGem(NES*), where the LEALE SLAL motif was mutated to PEALESPAS (changes are underlined), was enriched in the nuclei of all transfected cells (Fig. 3K). Geminin fused with a simian virus 40 T-antigen NLS (PPKKKRK) (15) became localized exclusively in the nucleus (GFP-NLS-cGem) (Fig. 3L). Geminin with the highly conserved destruction box deleted (GFP-cGemDel) was enriched in the nuclei of transfected cells (Fig. 3M and N). An N-terminal myristoylation signal (Myr; MGSSKSKPKDPSQR) adds a 14-carbon-lipid chain to the N terminus of the fusion

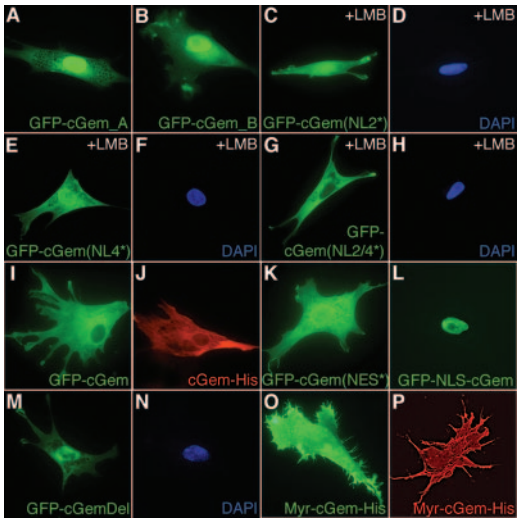

FIG. 3. Subcellular localization of geminin variants encoded by plasmid. Proteins were detected by GFP autofluorescence (A to I and K to N) or immunocytochemistry (J, O, and P) with fluorescence (A to O) or confocal (P) microscopy.

protein and thus directs a protein to the inner plasma membrane (1). After transfection into CEFs, fluorescence microscopy showed the anchorage of Myr-cGem-His to the cytoplasmic membrane (Fig. 3O), which was further confirmed by confocal microscopy indicating the accumulation of the protein on the membrane (Fig. 3P).

Regulation of chromatin loading of MCM by nuclear-cytoplasmic shuttling of geminin. To investigate the involvement of nuclear-cytoplasmic shuttling in regulating geminin's function as a cell cycle inhibitor, CEFs were transfected with different, modified forms of geminin or treated with LMB. Afterwards, the chromatin phases of the manipulated cells were isolated, and the loading of MCM3 onto chromatin was analyzed by Western blotting, with histone 2B as a control (Fig. 4A). Compared to untransfected or GFP-transfected cells, overexpressed GFP-cGem inhibited the loading of MCM3 onto chromatin. It is noteworthy that GFP-cGem is expected to shuttle like the endogenous geminin and thus to be present temporarily in the nucleus. As described above, the nuclear export of geminin is blocked by a mutated or absent NES, by a removal of the destruction box, or by an NLS fusion. Thus, overexpression of GFP-cGem_B, GFP-cGem(NES*), GFP-cGemDel, or GFP-NLS-cGem inhibited the loading of MCM3 protein like the wild type. On the contrary, the nuclear import process is blocked by mutated import signals or by myristoylation. Thus, overexpression of GFP-cGem(NL2/4*) or Myr-cGem-His proteins could not inhibit MCM3 loading (Fig. 4A). If the nuclear export of endogenous geminin was blocked through the inhibition of Crm1 by LMB, the nuclear retention of endogenous geminin protein led to a repression of proper chromatin loading of MCM3 (Fig. 4A). These results demonstrate that the nuclear import process is necessary and sufficient for inhibition of MCM loading by geminin, whereas the nuclear export process of geminin in G_1 phase inactivates the geminin protein as a cell cycle regulator.

To make sure that the inhibition of MCM3 loading by LMB is due to the blocking of nuclear export of geminin and not some other target proteins of Crm1, CEFs were treated with siRNA against geminin (siGem) (23) and with LMB, independently or sequentially, before the loading of MCM3 protein was measured (Fig. 4B). As indicated above, the loading of MCM3 onto chromatin was inhibited by treatment with LMB. Nevertheless, although the elimination of endogenous geminin protein by siRNA resulted in an overloading of MCM3 onto chromatin, subsequent treatment of LMB did not lead to a reduction of MCM3 loading. Therefore, the inhibition of MCM3 loading by LMB is due to the blocking of nuclear export of geminin, since this is not the case in the absence of geminin. Taken together, these data indicate that overex-

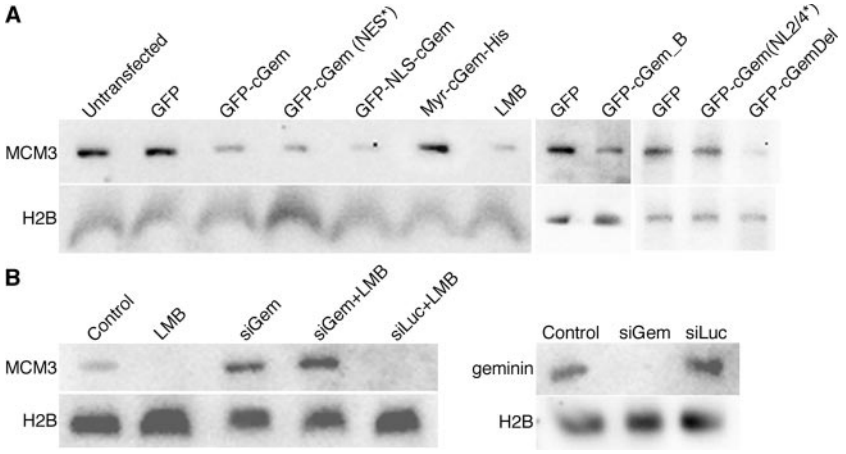

FIG. 4. Regulation of chromatin loading of MCM3 by nuclear-cytoplasmic shuttling of geminin. (A) Inhibition of MCM3 loading by nuclear- but not cytoplasmic- or membrane-associated geminin. Note that in contrast to untransfected or GFP-transfected control cells, overexpressed wild-type GFP-cGem, as well as truncated GFP-cGem_B, nuclearly retained GFP-cGem(NES*), and GFP-NLS-cGem, but not cytoplasmic GFP-cGem(NL2/4*) or membrane-associated Myr-cGem-His, inhibits chromatin loading of MCM3. If the nuclear export of endogenous geminin is inhibited by LMB, chromatin loading of MCM3 is repressed. (B) The unloading of MCM3 by LMB is due to blocking of the nuclear export of geminin. The left panel shows that MCM3 fails to dissociate from chromatin in the absence of geminin (siGem), regardless of the subsequent presence of LMB (siGem+LMB). The specific knockdown of geminin by siGem is shown in the right panel. H2B, histone 2B.

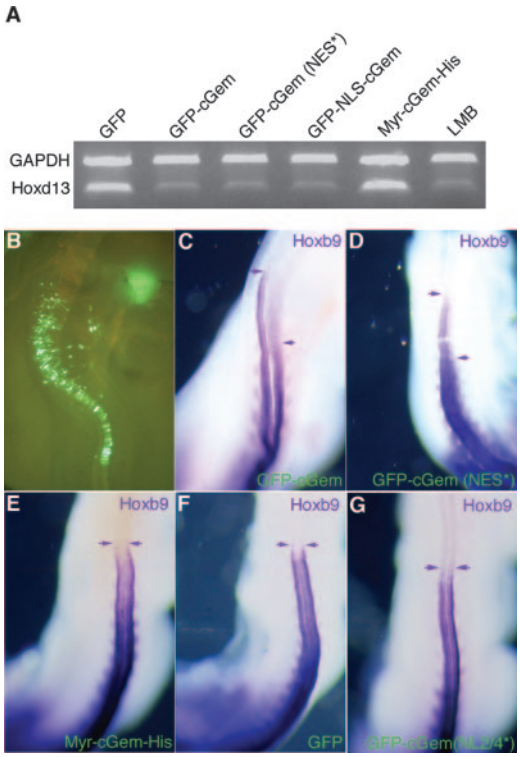

FIG. 5. Regulation of *Hox* gene transcription by nuclear-cytoplasmic shuttling of geminin. (A) Regulation of *Hoxd13* transcription by nuclear-cytoplasmic shuttling of geminin. Note that in the cultured mesenchymal cells, treatment with overexpressed wild-type or nuclearly retained geminin as well as LMB, but not overexpressed Myr-cGem-His, inhibits *Hoxd13* transcription to low levels compared to the effects of overexpressed GFP. (B to G) The involvement of nuclear-cytoplasmic shuttling of geminin in regulating *Hox* gene transcription in embryos. Successful electroporations are indicated by GFP expression (B). Note that overexpressed wild-type GFP-cGem or nuclearly retained GFP-cGem(NES*), but not Myr-cGem-His, GFP-cGem(NL2/4*), or GFP alone, can posteriorly shift the anterior transcription boundary of *Hoxb9* (arrows) in the right side of the neural tube.

pressed avian geminin can inhibit the loading of the MCM complex onto chromatin if the geminin is either allowed to shuttle or forced into the nucleus. However, a repression of MCM loading is no longer observable if the overexpressed geminin is kept in the cytoplasm. The dependence on LMB indicates an involvement of Crm1 in the inactivation of endogenous geminin as an inhibitor of MCM loading.

Regulation of *Hox* gene transcription by nuclear-cytoplasmic shuttling of geminin. Geminin represses *Hox* gene transcriptions through the polycomb pathway (23). The involvement of nuclear-cytoplasmic shuttling of avian geminin in regulating *Hox* gene transcription was studied in cultured mesenchymal cells prepared from wing buds of stage HH19 chick embryos. After transfections with different modified forms of geminin or a treatment with LMB, the transcription levels of the *Hoxd13* gene in the mesenchymal cells were analyzed by RT-PCR, with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as an internal control (Fig. 5A). If wild-type GFP-cGem, nuclear-retained GFP-cGem(NES*), or GFP-NLS-cGem was overexpressed in the mesenchymal cells, the *Hoxd13* transcription was repressed to similar low levels. By contrast, overexpression of Myr-cGem-His, which cannot be imported into the nucleus, did not affect *Hoxd13* transcription. Moreover, if the nuclear export was blocked by LMB, the nuclearly retained endogenous geminin protein reduced *Hoxd13* transcription to a lower level. These data indicate that the nuclear import process is essential and sufficient for geminin to inhibit *Hox* gene transcription, whereas the nuclear export process is necessary to inactivate geminin as a *Hox* gene repressor.

To further analyze the role of nuclear-cytoplasmic shuttling of geminin in regulating *Hox* gene transcription in embryos, different expression vectors for modified forms of geminin were coinjected with GFP expression plasmids into the neural tubes of stage HH9-11 chick embryos, electroporated, and consequently overexpressed in the right side of the neural tube. Forty-eight hours after electroporation, the GFP expressions were assayed (Fig. 5B) and the *Hoxb9* transcriptions of GFP-positive embryos were examined by whole-mount in situ hybridization (Fig. 5C to G). In the embryos with ectopic GFP-cGem or GFP-cGem(NES*), the anterior transcription boundary of *Hoxb9* was posteriorly shifted the length of one to four somites on the right, electroporated side of the neural tube. By contrast, there was no posterior shift of endogenous *Hoxb9* transcription in the embryos receiving GFP-cGem(NL2/4*), Myr-cGem-His, or only GFP. Taken together, these data demonstrate that avian geminin is able to repress *Hox* gene transcription as long as it retains the capability to shuttle into the nucleus.

DISCUSSION

Geminin has been isolated from many metazoans in recent years, including *Drosophila* spp., Medaka fish, *Xenopus* spp., mouse, and human, and intensively studied. This paper identifies avian geminin, suggesting its structural and functional protein sequence conservation with other organisms by its function of inhibiting the loading of the MCM complex onto chromatin and participating in developmental patterning processes. More intriguingly, we demonstrate a novel mechanism for regulating the availability of geminin in chick cells by cell cycle-dependent nuclear-cytoplasmic shuttling (Fig. 6). In G_1 phase, avian geminin is localized in the cytoplasm, where it remains unavailable for nuclear functions. We observed that in aphidicolin-treated cells, geminin is still cytoplasmic, although already some origins have been fired. Apparently, geminin is not immediately available for an inhibition of rereplication when origins become active. In early S phase, other redundant mechanisms may play the major part in the inhibition of DNA rereplication instead of the presence of nuclear geminin. Geminin shuttles into nuclei from the cytoplasm in the S phase and is exported back to the cytoplasm only upon transition from M to the next G_1 phase. On one hand, nuclear geminin inhibits loading of the MCM complex onto chromatin to prevent DNA rereplication. On the other hand, nuclear geminin is available to repress *Hox* gene transcription. After mitosis, geminin remains unavailable in the nucleus during the next G_1 phase, and thus, nuclear functions like the loading of the MCM complex and *Hox* gene transcription are ensured. The cytoplasmic localization of geminin during G_1 is the consequence of exportation from nuclei by the exportin protein Crm1, a process that can be specifically blocked by LMB. Nuclear-cyto-

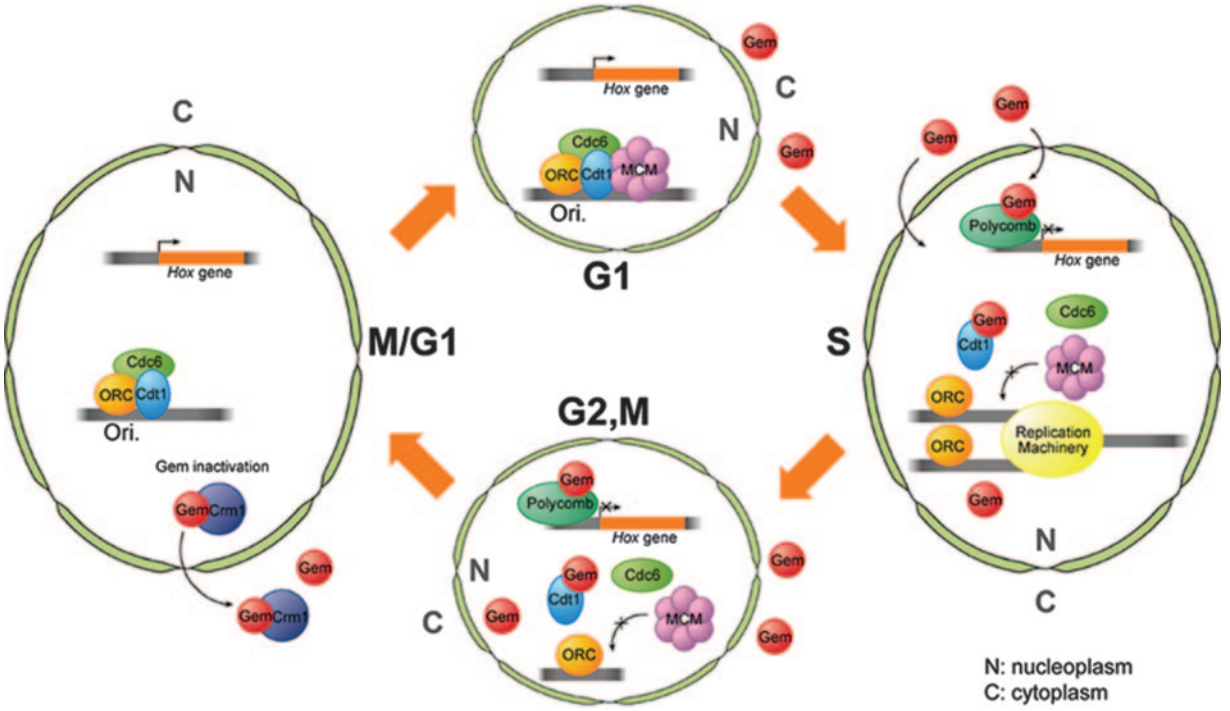

FIG. 6. Model for cell cycle-dependent nuclear-cytoplasmic shuttling of avian geminin. For discussion, please see the text.

plasmic shuttling was previously identified as a regulatory mechanism for some cell cycle regulators and developmentally important proteins, such as the tumor suppressor p53 and histone deacetylase HDAC5 (8, 26, 34). In addition, cell cycle-dependent subcellular localization of cyclin B1 is also regulated by Crm1 (13, 41). Here, we demonstrate not only a cell cycle- and Crm1-dependent nuclear-cytoplasmic shuttling of avian geminin but also a strict coordination between this shuttling and its functions.

Endogenous geminin protein in *Xenopus*, murine, and human cells was originally found exclusively in nuclei (3, 25, 31). The basic regions NL1, NL2, NL3, and NL4 (Fig. 1A) were shown by mutational analysis to be involved in nuclear localization of Geminin proteins in various species. NL1/NL2 (3) and NL2/NL3 (5, 43) were identified as essential bipartite NLS in *Xenopus*. NL4 is essential for nuclear localization of human geminin (5). Our study demonstrates that in the chick, where NL3 is not conserved, either NL2 (possibly in conjunction with NL1) or NL4 is sufficient for nuclear import. In analogy to the findings in human cells (43), the importance of NL4 in chick geminin may lie in an interaction with Cdt1, which could be responsible for transporting chicken geminin into the nucleus. More recently, a cytoplasmic localization of geminin was described for some cells of the *Xenopus* blastula and for murine P19 cells undergoing differentiation. The conservation of NES sequences and a further analysis of transfected human cells suggested that nuclear-cytoplasmic shuttling may be involved in the regulation of geminin activity also in other species (5). It remains to be seen whether this occurs with dependence on the cell cycle.

Geminin was originally proposed to be inactivated at the end of mitosis by APC-dependent degradation in mammalian cells (25), which was further confirmed by immunocytochemistry with the human osteosarcoma cell line U2-OS. In *Xenopus*, a significant proportion of geminin was found to escape from proteolysis at the end of mitosis (14). Nevertheless, the surviving population of geminin loses its affinity for Cdt1 and does not inhibit DNA replication licensing in the next G_1 phase. A scrutiny for the inactivation mechanism of the survival population of geminin revealed that transient ubiquitination of geminin without proteolysis is essential for geminin inactivation and replication origins to become licensed (21). All these findings propose that different mechanisms can be applied to regulate geminin in different organisms and even synergistically in one organism. We demonstrate here a novel mechanism for regulating the functions of geminin by nuclear-cytoplasmic shuttling. However, since the destruction box sequence is strongly conserved (Fig. 1A), we do not exclude the possibility that a part of avian geminin is degraded by APC-dependent proteolysis at the end of mitosis. Furthermore, some important and interesting questions are still open. For instance, why do birds adopt this special mechanism to regulate geminin? Does the cytoplasmic geminin obtain special regulatory functions there? What is the evolutionary significance of the mechanism? Cell cycle-dependent nuclear-cytoplasmic shuttling of cyclin B1 is controlled by combinatorial phosphorylation at multiple sites in different cell cycle phases (42). Therefore, another important question is, what is the key switch leading to the interaction between geminin and Crm1 and thereby to the nuclear export at the end of mitosis? Regarding this question, we have three hypotheses. First, some modifications, like phosphorylation or ubiquitination, could occur upon exit of mitosis, which results in conformational change of geminin proteins and thus an exposure of NES

sequence to Crm1. These modifications could also dissociate geminin from other proteins, like Cdt1, Hox, or Six3, making it available for Crm1. Second, some interaction partners of geminin, like Hoxc10, are targets of APC and their presences are also cell cycle dependent (10). It could be that the degradation of interaction partners at the end of mitosis leads to release of the geminin protein for Crm1. Third, it could be that the Crm1 protein itself is sequestrated until the end of mitosis. Among these three hypotheses, the first one appeared to be most likely, since the deletion of the destruction box resulted in the nuclear enrichment of geminin (Fig. 3M and N). A destruction box-dependent, and therefore APC-mediated, modification may represent the switch that exposes geminin's NES to Crm1 and thus triggers the export from the nucleus.

In summary, our results suggest the regulation of geminin functions by Crm1-dependent nuclear-cytoplasmic shuttling. This shuttling is coordinated with different cell cycle phases and regulates the availability of geminin in the nucleus as an inhibitor of both MCM loading and *Hox* gene transcription, ensuring proper cell cycle and developmental processes.

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