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News and Commentary

Role of geminin: from normal control of DNA replication to cancer formation and progression?

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The normal cell proliferation involves the passage through a series of finely regulated phases that are known as cell cycle checkpoints. In order to carry out a correct replication, the eukaryotic cell undergoes to a series of events ensuring that each copy of duplicated chromosome exactly segregates in each of daughter cells.[1,2]

A main critical control of the cell cycle is to ensure that the DNA replication takes place and that it is followed by mitosis, once for each cell cycle. Alterations in the expression of genes that regulate the cell cycle can lead to malignant transformation and tumor progression by perturbing cell proliferation and/or genomic stability.[3,4]

The normal progression through the cell cycle is a critical step for the survival of eukaryotic cells, in which the initiation of DNA replication is under the tight control of several factors that ensure the exact duplication of chromosomes in S phase and their subsequent segregation in phase M. The strict regulation of the cell cycle combined with the presence of a *licensing* phase, in which the chromatin becomes licensed to be replicated, leads to the existence of a single DNA replication start site for each round of replication.[5]

In this complicated network of signals regulating the cell cycle and maintaining genome integrity, the geminin protein is considered one of the main players. However, if the geminin acts as oncosuppressor gene or as a proto-oncogene is still an open question.

In this article, we will report the mechanism by which geminin controls that the huge quantity of eukaryotes DNA, distributed over multiples chromosomes, is replicated once and once only for each cell cycle. Moreover, we will open and discuss an important question: what role does geminin play in cancer formation and progression? Does geminin act as an oncosuppressor, as a proto-oncogene or exhibit both roles?

Geminin in the Licensing

The replication of eukaryotic genome is a complex process strictly regulated by an intricate intracellular and extracellular signaling network, which controls different phenomena such as cell proliferation, differentiation and apoptosis.[6,7] All these processes have a common function: the regulation of the initial phase of DNA replication. During this event, specific proteins sequentially assemble onto the replication origin by forming specific replicative complexes (pre-RC), which allow for the chromatin to be competent (*licensed*) for the next DNA replication step of the cell cycle.[8]

Geminin is a 25 kDa nuclear protein that functions by inhibiting DNA replication.[9] During specific phases of the cell cycle, geminin is able to bind to Cdt1 protein and inhibits pre-RC formation.[10] In eukaryotes, the origins of replication are bound by a complex of six proteins, evolutionally well conserved among different species.[11,12] These proteins constitute the origin recognition complex (ORC) (1–6), a complex of proteins essential for the initiation of DNA replication, which was first identified in *Saccharomyces cerevisiae* as protein that bound to the core ARS consensus sequence.[13] The ORC complex constitutes the substrate on the chromatin for the assembly of other replication initiation factors such as Cdc6, an ATPase family member, and Cdt1.[14,15] The recruitment of these proteins induces a chromatin structural change that promotes the loading of the mini-chromosome maintenance proteins (MCMs 2–7) onto the ORC/chromatin complex.[16] The MCM proteins have a similar helicase activity and are able to unwind the DNA double helix in order to make it more accessible to the action of different polymerases.[17] During the G1 phase of the cell cycle, the aforementioned proteins are sequentially recruited on the origins of replication and form the pre-replicative complex (pre-RC).[18] At the G1/S transition, the licensed origins are activated by the activity of cyclin E/CDK2, cyclin A/CDK2 and Cdc7-Dbf4 kinases and by the recruitment of MCM10 protein.[19,20] Cdt1 cooperates with Cdc6 to promote the DNA initiation by regulating the formation of pre-RC through the recruitment of MCMs on the chromatin associated with ORCs.[21,22]

The activation of the pre-RC complex takes place with the release of Cdc6 and the recruitment of Cdc45.[11,23] This latter allows DNA polymerase to bind to the activated pre-RC complex and initiate RNA-primed DNA synthesis at the particular origin.[24,25] Once DNA synthesis is initiated, the MCM proteins (2–7) remain associated with replication forks, probably providing the replicative helicase activity; once DNA synthesis is completed, they dissociate from chromatin and the pre-RC complex is disassembled to ensure that the origin cannot 'fire' again during that cell cycle.[26] The re-firing of origins in the same cell cycle is also prevented by the cyclin-dependent kinase. In fact, the increasing CDK activity

inactivates Cdc6, MCMs and Orc2 for preventing re-replication. Following, the Cdt1 inhibition by geminin and the Cdc6 phosphorylation prevent the assembly of new pre-replication complexes during S phase.[11,27] The formation of new pre-RC complexes will occur that both geminin and mitotic cyclins are degraded by the anaphase-promoting complex (APC) during mitosis. In fact, the degradation of geminin by the APC allows Cdt1 to reload the MCM proteins on the chromatin and rebuild a new replication complex[9] (Figure 1).

Geminin–Cdt1 Interaction

Recent studies have reported the geminin structure and explained the nature of geminin–Cdt1 interaction by highlighting the structural importance of the coiled-coil domain of the geminin dimer for the inhibition of pre-RC assembly.[28,29] It is known that geminin is a potent inhibitor of origin assembly and re-replication in multicellular eukaryotes.[9,10] During the S and G2 phases, geminin blocks the binding of the MCM complex to the replication origins mainly by interacting with Cdt1, which is active in G1 phase to establish functional pre-RCs.[10,16,30–32]

Structurally, the geminin protein contains a dimerization domain, an N-terminal basic region and a C-terminal tail rich in

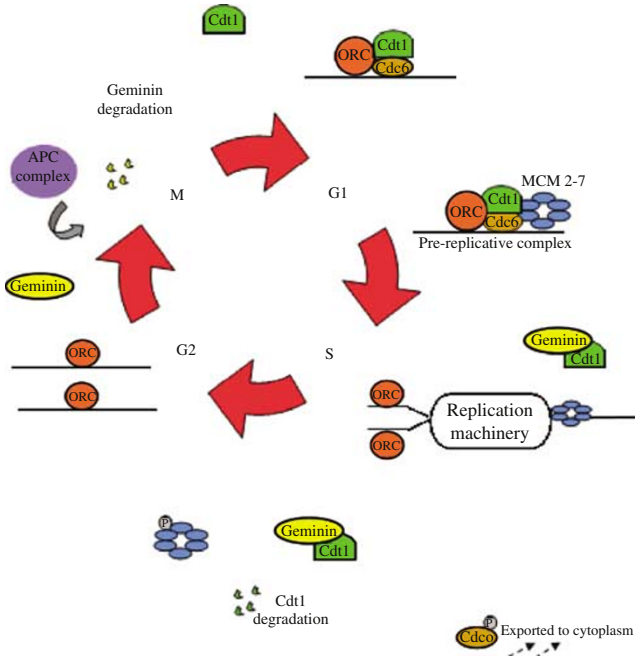

Figure 1 Geminin role in DNA replication. During G1 phase, the origin recognition complex (ORC), which constitutively binds to the origins of replication, functions as a substrate for the replication factors Cdc6 and Cdt1. The binding of Cdt1 induces the recruitment of the hexameric minichromosome maintenance complex (MCM 2–7) to chromatin forming the pre-replication complex (Pre-RC). Once the DNA replication is started, geminin, whose expression is high during S phase, binds to Cdt1, whereas the MCM proteins and Cdc6 are phosphorylated by cyclin-CDKs activity and displaced from chromatin. Cdc6 is exported to the cytoplasm and geminin continues to bind Cdt1 until the DNA is replicated. In G2 phase, only ORC is bound to chromatin, and geminin expression levels are maintained high to ensure that inappropriate expression of Cdc6 and Cdt1 does not lead to aberrant licensing. The degradation of geminin by anaphase-promoting complex (APC) at the metaphase/anaphase transition, the dephosphorylation and the activation of replication factors, when the mitosis is completed, permits the initiation of a new round of DNA replication

acidic amino acids, which are poorly conserved among species.[9,33] Specifically, the amino-terminus region of geminin contains a short sequence, highly conserved in *Xenopus*, which is highly homologous with the consensus sequence of the mitotic cyclins destruction box. Moreover, the core of geminin protein contains two regions. One of these is a rich basic amino-acid region (50–116) that represents the bipartite nuclear localization signal (NLS) and/or the region of ubiquitin attachment (destruction box) for the ubiquitination and destruction of geminin during mitosis. The other region contains a coiled-coil domain and represents the putative protein–protein interaction's region.[33] The internal coiled-coil domain of geminin consists of at least five heptad repeats. Geminin dimerizes through its internal coiled-coil domain and interacts with Cdt1 by a specific Cdt1-binding site, which is located near to the coiled-coil domain.[33] Furthermore, it has been reported that the sequences between the NLS and the Cdt1 binding-domain are not required for the inhibition.[33]

Recently, it has been shown that human geminin can bind to Cdt1 by its central region that contains a dimerized and negatively charged coiled-coil domain utilizing, as well as an adjoining region to interact with the N-terminus 100 residues of Cdt1, in order to inhibit DNA re-replication.[28]

Recently, the crystal structure of a mouse geminin-cdt1 complex has been described.[34] This work has added further information regarding the distinct roles of geminin in the direct binding to Cdt1 (through its N-terminal part) and in the inhibition of the MCMs binding to Cdt1 (through its C-terminal part), as well as the structural importance of the coiled-coil region for the inhibition of pre-RC assembly.[34] Although the coiled-coil region is necessary for geminin functions, as well as geminin dimerization, it is not sufficient to inhibit DNA synthesis: extra sequences at the C-terminus of the coiled coil are required to give a functional domain.[29]

Geminin–Cdt1: An Important Balance

The importance of geminin–Cdt1 interaction in the prevention of DNA re-replication was recently highlighted in Cdt1 overexpression experiments in human cells with inactive p53.[35] Overexpression of Cdt1 not only promotes re-replication but also stimulates ATM/ATR/Chk2 DNA damage-induced checkpoint pathway. This pathway activates p53 as an additional tool of mammalian cells to prevent re-replication, through the induction of cdk2 inhibitor p21.[35] Interestingly, geminin depletion causes partial DNA over-replication in HCT116, U2OS cancer cells and diploid fibroblasts TIG3 human cells, accompanied by an activation of a different ATR/ATM checkpoint pathway.[36] Significantly, this over-replication did not occur when Cdt1 was also repressed.[36]

Interestingly, it has been described that geminin inactivation causes centrosome overduplication without passage through mitosis in human cancer cells. The geminin-depleted cells show abnormal and multipolar spindles, when driven into mitosis by checkpoint abrogation, suggesting a new general role for geminin in coordinating the centrosome duplication besides licensing inhibitor of DNA replication.[37]

Therefore, it has been proposed that geminin becomes necessary as a regulatory factor only when the concentration of Cdt1 remains high during S phase.[38] Geminin ensures

basal levels of Cdt1 during S phase and its accumulation during mitosis.[39] In addition, geminin can function as a negative or positive regulator of pre-RC formation in human cells and protect Cdt1 from proteasome-mediated degradation by inhibiting its ubiquitination.[39]

Geminin: Multiple Functions in Development and Differentiation

In addition to inhibiting DNA replication, geminin plays an important role in the development and differentiation of embryonic cells.[40] It has been reported that geminin is involved in the formation of the neural tube, by inducing uncommitted ectodermal cells to differentiate into nervous tissue in *Xenopus*.[40] Moreover, it has been indicated that geminin is able to induce the entry of cells into mitosis by regulating Chk1 kinase.[41–43] In *Drosophila*, geminin displays all the features seen in *Xenopus* and human. In fact, it is able to block the MCMs' recruitment on the pre-RC by binding to Dup (Doubleparked), a Cdt1 homolog; in addition, geminin is expressed in both S and G2 phases and it is degraded during metaphase/anaphase transition.[44] Moreover, overexpression of geminin can induce neural differentiation.[44]

Interestingly, it has been found that geminin is also involved in the regulation of Hox homeobox proteins through direct and polycomb-mediated interactions, thereby preventing the Hox proteins from binding on to their target DNA sequence.[45] Moreover, it has been shown geminin can inhibit Hox-dependent transcription activation of target genes by perturbing the axial segmentation pattern in chicken embryo.[45]

In medaka fish embryo, geminin is able to inhibit eye formation by binding Six3, a transcription factor involved in eye development.[46] Geminin regulates the transcription of the Six3 gene by inhibiting its activity.[46] In addition, Six3 antagonizes geminin activity by acting like a Cdt1 competitor and displacing geminin from Cdt1.[46] In this way, genes that are involved in regulating development also control cell proliferation by directly inhibiting the interaction of geminin with Cdt1. Moreover, not only geminin is able to antagonize the function of Six3 in gene transcription without interfering with its DNA-binding activity, but it also antagonizes the Hox function by displacing it from the genes that it targets and/or by regulating *Hox* gene expression through the interaction with specific polycomb gene pathways.[47]

Interestingly, it has been reported that geminin is able to regulate neuronal differentiation by antagonizing the activity of Brg1, a catalytic subunit of the SWI/SNF chromatin remodeling complex.[48] By its C-terminus domain, geminin binds Brg1, blocks the association of Brg1 with proneural bHLH proteins and, thus, prevent the transcriptional activation of their target genes. Geminin could act by regulating the interaction between SWI/SNF complex and transcription factors and maintaining the undifferentiated state of neural progenitor cells.[48] The overexpression of geminin defective in Brg1-binding domain, without affecting the DNA replication, prevents the neuronal differentiation, whereas reduction of geminin activity causes premature neurogenesis. In this point of view, geminin appears as a transcriptional regulator that acts by modulating the activities of SWI/SNF chromatin-remodeling complex for Ngn/NeuroD-driven neurogenesis.[48]

Recently, GMN-1, a geminin homologue, has been identified in *Caenorhabditis elegans*. GMN-1 is able to associate with *C. elegans* CDT1, to inhibit the interaction between mouse Cdt1 and Mcm6, and to bind *in vitro* homeobox proteins (NOB-1 and CEH-32) in order to control the development and differentiation in this organism.[49] However, the geminin–cdt1 interaction is conserved through the metazoans, although the affinity of GMN-1 for CDT1 is rather weak compared to *Xenopus* or mouse system.[49] Probably, during the evolution, geminin could be evolved in these taxa to regulate the proliferation and differentiation by directly interacting with cdt1 and homeobox proteins or by an unknown cell cycle regulator.

Who Does Control Geminin Transcription?

The mechanism that controls the transcriptional regulation of the geminin gene is poorly understood. However, it has been found that geminin expression is tightly cell cycle regulated in normal cells and that newly synthesized geminin is subject to a turnover during S phase with a half-life of 3–4 h.[50] Interestingly, it has been shown that geminin and Cdt1 are targets for E2F transcription factors and that their expression is mediated by the activation of the pRb/E2F pathway.[51,52] Rb can exert its influence on geminin gene expression through highly conserved E2F sites, located downstream to the geminin promoter gene, in proximity of the first exon. Geminin and Cdt1 are activated by E2F4, and modulation of a pRb/E2F pathway is directly linked to the regulation of geminin and Cdt1 promoter activity. As E2F1-induced activation of human Cdt1 gene transcription is suppressed by pRb, but not by the other retinoblastoma family members p107 or p130, and as its E2F4-induced activation is suppressed by pRb, p107 and p130, it has been proposed that the disruption of pRb or activation of certain members of the E2F family can lead to overexpression of the geminin and Cdt1 proteins.[51] Therefore, it has been proposed that Rb is required to maintain appropriate levels of geminin and that the trascriptional control of geminin by Rb could limit *de novo* synthesis of geminin in G1, thereby preventing the need to alter additional geminin after the transition into the G1 phase.[52]

As pRb1/p105, pRb2/p130 and p107 proteins are important regulators of cell cycle progression, and the geminin protein plays an important role in maintaining the genome integrity as well, it is our opinion that an unknown feedback loop between Rb family and geminin proteins regulates the geminin transcription.

Geminin and Cancer: Does Geminin Act as an Oncosuppressor, as a Proto-oncogene or Exhibit Both Roles?

Several studies have reported that exogenous geminin blocks DNA replication and inhibits progression through the cell cycle.[9,30,53,54] The ability of geminin to suppress initiation of DNA replication in *Xenopus* egg extracts led to the hypothesis that this protein acts as an inhibitor of cell proliferation and might be a tumor suppressor gene.[42,36] However, geminin

expression in normal and cancer cells does not correlate with a decrease in cellular proliferation. Immunohistochemistry and immunoblotting for geminin revealed that, except in testis, where its expression is high, this protein is absent in organs with minimal proliferation activity (heart, nerves, prostate, kidney, lung and scheletric muscle) and it is specifically expressed in proliferating lymphocytes, male germ cells and epithelial cells.[55–57] Interestingly, geminin is frequently overexpressed in human primary colon, rectal and breast tumors, in a subset of non-small-cell lung carcinomas, in B-cell lymphoma and in cancer cell lines of different histogenic origin and its upregulation correlated with high proliferative activity of tumor cells,[58–62] suggesting that this protein could exhibit oncogenic activity. Although being initially proposed as a tumor suppressor gene, geminin does not appear to exert an antiproliferative effect in these tumors but its role is supportive to consider geminin as a marker of proliferation.[63,64] In oligodendroglial tumors, geminin does not behave as an inhibitor of cell proliferation and its expression in higher grade tumors is strongly correlated to proliferation.[65] In addition, in breast cancer tissues, high levels of geminin strongly correlate with adverse clinical outcome.[62]

Recently, it has been shown that the overexpression of wild-type geminin in breast, osteosarcoma (U2OS), 293T, HeLa and colon cancer (HCT116) cells, did not produce a cell cycle block.[58,55] On the other hand, transfection of a mutant non-degradable form of geminin was shown to induce arrest of cell cycle progression and proliferation in osteosarcoma and breast cancer cell lines,[66] as well as in a colon cancer cell line.[67] Moreover, it has been reported that alteration of the 'destruction box' sequence in the geminin gene causes stable protein accumulation, inducing inhibition of DNA replication and a decrease of MCMs' chromatin recruitment, consequently leading to cell cycle arrest.[67] This arrest does not occur by overexpression of a wild-type geminin protein, as it is likely degraded during G1 phase by the APC complex with the same mechanism shown for the endogenous geminin.[9,67]

In addition, several sets of conflicting data exist regarding the silencing of geminin in different cancer and normal cell lines. It has been shown by Melixian *et al.*, that depletion of geminin does not lead to DNA over-replication in HeLa cells as seen in HCT116, US2OS and normal fibroblast.[31,36,38]

Further information is necessary to better delineate the role(s) played by geminin in the regulation of cell cycle machinery in both normal and cancer cells. In our opinion, in transformed cells whose proliferation has escaped the control of important cell cycle regulators such as geminin protein, cell cycle progression may occur independently of what geminin may do to maintain a correct pattern of DNA replication and to control specific mechanisms regulating cell cycle, thus, acting to promote tumor progression rather than as a classic tumor suppressor.

Concluding Remarks

The cell cycle is an extremely fine-tuned process which responds to the specific needs of any specific tissue or cell. Several mechanisms ensure that DNA replication is followed by mitosis and occurs only once per cell cycle. However, genetic and epigenetic events can occur that alter the mechanisms of cell cycle control, promoting genome instability, chromosome polyploidy and/or aneuploidy and, finally, tumor formation and progression.[68] In eukaryotes, geminin interacts with Cdt1 and regulates its activity to prevent DNA replication during inappropriate times of the cell cycle. Currently, the mechanisms by which geminin inhibits Cdt1 are not completely clear as well as the mechanisms regulating geminin transcription.

Taken together, further information is needed to better understand the mechanisms of geminin action as well as its interaction with others cell cycle regulators. Future studies could clarify the exact role of this protein in cell cycle deregulation, observed in tumor cells, and also assess if geminin protein could be considered as a possible tumor marker.

This article opens an important debate regarding the complexity by which the geminin function could appear so different when different cellular systems, as well as cancer or normal cells, are investigated.

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