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Geminin organizes the molecular platform to balance cellular proliferation and differentiation

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1. ABSTRACT

Geminin was originally identified as an inhibitor of replication initiation, but is now known to play multiple roles in several fundamental cellular processes including proliferation, differentiation, development and transcriptional regulation. Recently, the functional properties of geminin have been further characterized by identifying geminin binding partners. To gain a broader molecular view of geminin's role in the control of cellular functions, we performed yeast two-hybrid screening. In this review, we will describe several novel binding partners of geminin, particularly those involved in the DNA repair process performed by TopBP1, as well as some unexpected roles of geminin in various cellular events.

2. INTRODUCTION

Geminin was first identified as a 25-kD nuclear protein that inhibits the timing of DNA replication and is degraded during the mitosis phase of the cell cycle (1). The mitotic degradation of geminin is due to ubiquitination by the anaphase-promoting complex (APC/C). The destruction of geminin depends on the presence of an N-terminal destruction box motif, which is also present in cyclin B (2,3). This regulatory control permits geminin to act in a cell-cycle-dependent manner. Geminin inhibits the initiation of replication by interacting with replication initiation factor Cdt1 and preventing the incorporation of licensing factors, like minichromosome maintenance (MCM) 2-7 proteins, into the prereplication complex (1,4-

16). The balance of the geminin-Cdt1 association establishes the timing of DNA replication initiation and controls cell cycle progression (17-23). In *Xenopus laevis* egg extracts, the cyclin-dependent kinase (CDK)-dependent activation of the APC/C results in the transient polyubiquitination of geminin (24). This ubiquitination triggers geminin inactivation without requiring ubiquitin-dependent proteolysis and is essential for the licensing of replication origins. Therefore, geminin is regulated by CDK as part of the regulation of cell cycle progression.

Geminin is now regarded as a modulator in early embryonic patterning because of its direct interaction with Six3 and Hox homeodomain proteins, thereby inhibiting their functions; it also is involved in a transient association with the Hox repressive Polycomb complex of genes (25-27). In addition to the classical view described above, geminin is uniquely involved in many other cellular processes. In this review, we will discuss and summarize recent research related to geminin. Moreover, we will present several novel binding partners of geminin that were identified using yeast two-hybrid screening. Such information should expand the value of geminin in basic molecular research and also in clinical research, possibly enabling geminin to serve as a tool for cancer diagnosis or enabling the development of therapeutic agents based on the structure of geminin or geminin interactors.

3. BEYOND DNA REPLICATION

3.1. Mitosis and the cell cycle

The cohesin complex is a central player in sister chromatid cohesion, a process that ensures the faithful segregation of chromosomes during mitosis. Scc2/Mis4, a HEAT-repeat-containing protein, is required for the loading of the cohesin complex onto chromatin, and the immunodepletion of Scc2/Mis4 impairs the association of the cohesin complex with the chromatin, leading to severe defects in sister chromatid pairing in subsequent mitosis. The loading of Scc2/Mis4 onto chromatin is inhibited in extracts treated with geminin (28). Indeed, geminin inactivation causes centrosome overduplication without passage through mitosis in human normal and cancer cells (29). Furthermore, the depletion of geminin from a diploid mammary epithelial cell line caused the cells to arrest in late G2/M phase, indicating that geminin is required for proper cytokinesis (30).

In *Xenopus* egg extracts, preventing DNA replication by the addition of geminin disturbs the binding of topoisomerase II (topo II) to chromatin, suggesting that topo II is tightly coupled with prior DNA replication (31). Topo II is required for mitotic chromosome condensation. Therefore, geminin might be involved in the regulation of mitosis in the cell cycle. As discussed later, we propose that topo II binding protein 1 (TopBP1) may act as a novel geminin interactor. Geminin is involved in the regulation of multiple steps of the cell cycle, including DNA replication, centrosome duplication, and chromosome segregation (32).

3.2. Development and transcriptional regulation

At the same time as the identification of geminin as an inhibitor of DNA replication, geminin was also implicated in the regulation of development. In *Xenopus* embryos, geminin overexpression causes the expansion of the neural plate at the expense of the adjacent neural crest and epidermis (33). Geminin may be a downstream target of the *Xenopus* Brachyury homologue, Xbra, and is required for normal formation of the posterior mesoderm (34). The involvement of geminin in neurogenesis has also been described in the development of *Drosophila* (35).

The exciting molecular finding that geminin has an important role in the control of development comes from experiments using yeast two-hybrid screening. Geminin associates with the homeobox-containing transcription factor Six3 to control the balance between proliferation and differentiation during early vertebrate eye development (36). Six3 efficiently competes with Cdt1 to bind directly to geminin. Geminin has also been shown to associate with the members of the Hox-repressing polycomb complex, the chromatin of Hox regulatory DNA elements, and Hox proteins (37). In the chick neural tube, geminin modulates the anterior boundary of Hoxb9 transcription. The interaction between geminin and Hox proteins prevents Hox proteins from binding to DNA, inhibits Hox-dependent transcriptional activation of reporter and endogenous downstream target genes, and displaces Cdt1 from its complex with geminin. These findings strongly suggest that geminin functions as a coordinator of developmental and proliferative control by competitively regulating transcriptional factors. In regard to neuronal differentiation, geminin is also known to bind to transcription factor AP4 and act as a repressor complex to negatively regulate the expression of target genes in non-neuronal cells and may contribute to the suppression of the precocious expression of target genes in fetal brain (38).

SWI/SNF chromatin-remodeling protein Brg1 has also been shown to interact with geminin (39). Brg1 has been implicated in cell cycle withdrawal and cellular differentiation. Geminin antagonizes Brg1 activity during neurogenesis to maintain an undifferentiated cell state (40). Recent studies have shown that the formation of the inner cell mass is prevented and that premature endoreduplication at 8 cells, rather than at 32 cells, occurs in geminin-deficient mice embryos, indicating an essential role of geminin in normal development (41,42).

3.3. Cancer biology

The possible participation of geminin in cancer biology was first proposed because of geminin's inhibitory action on the replication of Epstein Barr virus (EBV) from oriP (43), since the relation between EBV and cancer is well known. A hallmark of cancer is genome instability, which is mainly caused by alterations in the control of DNA replication and mitosis. Geminin, together with cyclin A, are required for the suppression of over-replication and for genome stability in *Drosophila* cells (44). Geminin is downregulated when endomitosis occurs during the terminal differentiation of mammalian megakaryocytes (45). A non-degradable form of geminin suppressed tumorigenicity and increased the level of p53 protein in human cells (46). The inactivation of geminin leads to rereplication in human normal and tumor cells

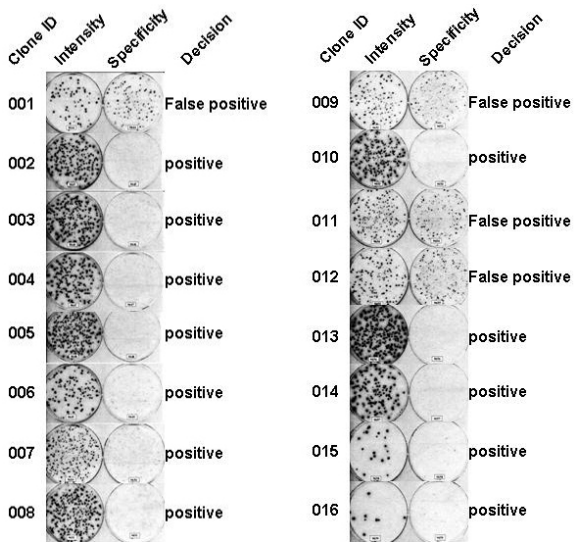

Figure 1. Representative pictures of beta-gal assay. To check the intensity of the beta-gal assay, geminin was used as bait and first-screening-positive clones were used as prey. Specificity was checked using a beta-gal assay; a mixture of six different genes known to bind non-specifically to any gene product was used as bait and first-screening-positive clones were used as prey. Positive and false-positive results were judged based on the intensity and specificity of the beta-gal assay.

independent of the functional status of p53, suggesting that geminin is required for the maintenance of genomic stability in human cells (47,48). The independent effects of proteolysis and geminin binding on Cdt1 inactivation after the onset of S phase are known to be sufficient to prevent rereplication (49). Recent data demonstrates that geminin is required for the prevention of rereplication in human normal and cancer cells; however, the relationship between geminin and p53 in the control of rereplication during the initial stage of tumor formation remains controversial (50-53).

Mutations of p53 or pRb genes are frequently seen in cancer cells. The inactivation of the pRb gene results in the continuous activation of transcription factor E2F1, on which the transcriptional regulation of geminin has been reported to depend (54,55). In addition, geminin is expressed specifically in proliferating lymphocytes and epithelial cells, indicating that geminin expression is positively correlated with cell proliferation, though geminin itself is a suppressor of cell proliferation (56). In a series of 55 oligodendrogliomas, geminin did not inhibit cell proliferation. Geminin is expressed in a higher proportion of cells in higher-grade tumors (57). Elevated geminin expression has been found to be a powerful independent indicator of an adverse prognosis in patients with invasive breast cancer (58,59). The combined analysis of licensing factors, including geminin, may contribute to improved treatment decisions and prognoses in patients with haematopoietic malignancies, such as B cell lymphomagenesis (60). Geminin is also overexpressed in colon carcinomas, and its expression is correlated with significant clinicopathological parameters of the disease (61). Geminin may represent a valuable tool in furthering our understanding of hepatocellular carcinoma progression from precursor lesions in a cirrhotic background (62). Accumulating evidence also suggests that geminin may be a novel molecular target for anti-cancer drug development (63-70).

4. EMERGING FEATURES OF GEMININ

4.1. Search for novel geminin partners

The two-hybrid system is a powerful technique for detecting protein-protein interactions using the well-developed molecular genetics of the yeast *Saccharomyces cerevisiae* (71,72). However, the most prominent problem is the contamination arising from the large number of false-positive results. We have used an improved technique called ProNet (Myriad Genetics) (73) that is extremely sensitive to weak interactions and eliminates nearly all false-positive results. To identify clones exhibiting geminin interactions, screenings were performed at the facilities of the Life Science Group, HITACHI, Ltd. (Kawagoe, Saitama, Japan). A bait construct expressing full-length geminin (amino acid residues 1-210; GenBank Accession Number NM_015895) fused to the C-terminus of the Gal4 DNA binding domain (amino acid residues 1-147) was transformed into yeast Mata strain PNY200. The bait alone did not induce transcriptional activation. Prey constructs expressing cDNA from several human breast and prostate cancer cell lines with polyA(+) RNA fused to the C-terminus of the Gal4 activation domain (amino acid residues 768-881) were transformed into the yeast MATa strain BK100, which contains multiple reporter system (74). PNY200 cells (bait) were mated with BK100 cells (prey), and diploid yeast cells were selected in the presence of 3 mM 3-amino-1,2,4-triazole for their ability to synthesize tryptophan (bait), leucine (prey), histidine (bait/prey interaction), and adenine (bait/prey interaction). By screening 3.3 X 10^7 clones, we succeeded in selecting 16 clones as first screening positive clones. These putative positive clones were reintroduced into the C-terminus of the Gal4 activation domain and tested again, as described above. The beta-galactosidase (gal) assay, a third reporter system, was performed as a final screening. Out of the 16 clones, 12 clones were selected after checking the intensity and specificity of the beta-gal assay (Figure 1). The intensity was evaluated using the results of a beta-gal assay employing geminin as the bait and first-screening-positive clones as the prey. On the other hand, specificity was tested using a beta-gal assay with a mixture of six different genes (gene names are confidential) known to bind any protein non-specifically as the bait and the first-screening-positive clones as the prey. As a result, four clones were excluded as false positives. Plasmids derived from positive yeast clones were recovered and sequenced using a primer corresponding to the Gal4 sequence.

4.2. Characterization of novel geminin partners

As shown in Table 1, two clones were proven to encode Cdt1 (GenBank Accession Number AF321125.1). This result strongly suggests that the yeast two-hybrid screening was successful. Seven clones were identical to a

Table 1. List of geminin-interacting proteins identified by yeast two-hybrid screening

PREY	CDS	REGION (AA)	CLONE ID
Cdt1	546	103~>259	015, 016
TopBP1	1435	106~295	007
LOC339287	614	142~336	002, 003, 004, 005, 008, 010, 014
GCC1	775	169~>369, 303~449	006, 013

The coding sequences (CDS) of the proteins and the regions encoded by the prey plasmids are shown. AA denotes the amino acid residues of the indicated protein. The clone IDs for each of the proteins correspond to those appearing in Figure 1.

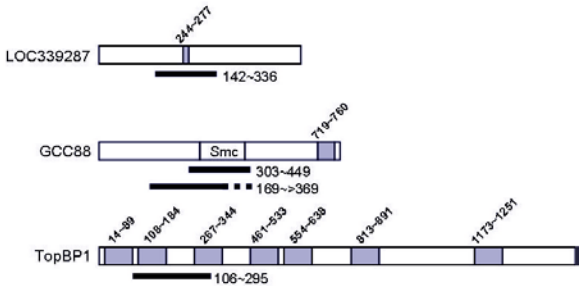

Figure 2. Schematic representation of the protein domains in the proteins identified using yeast two-hybrid screening. The central portion of the LOC339287 protein encodes a bZIP domain (grey box). GCC88 has an Smc region and a C-terminal GRIP domain (grey box). TopBP1 includes 7 BRCT domains (grey box) identified by a Pfam search. The numbers indicate the amino acid residues. The horizontal lines represent the regions that were shown to interact with geminin.

hypothetical protein, named LOC339287 (GenBank Accession Number XM_496217.1). Using a Pfam database search (score 0.65), we determined that the 244-277 amino acid residues of LOC339287 potentially encode a bZIP transcription factor-like region (Figure 2). We then constructed a GFP-fused LOC339287 expression vector and transiently transfected it into HeLa cells. GFP was exclusively detected in the nucleus (data not shown). The two clones were GRIP and coiled-coil domain containing 1 (GCC88; GenBank Accession Number BC078665). GCC88 is characteristic of extensive coiled-coil regions with the GRIP domain located at the C terminus (75). The GRIP domain is a targeting sequence found in a family of coiled-coil peripheral Golgi proteins. Indeed, GCC88 is directed to trans-Golgi network membranes by its C-terminal GRIP domain in a G-protein-dependent process (76). The translocation of geminin into the Golgi apparatus has never been reported. GCC88 may be translocated into the nucleus as a result of an alternative splice-out of the C-terminal GRIP domain or by binding to nuclear transporting proteins under certain conditions. By searching the Conserved Domain Database at NCBI, we found a Smc region around the central region of GCC88 (Figure 2). The Smc domain potentially encodes chromosome segregation ATPases (77). One clone corresponded to TopBP1 (GenBank Accession Number NM_007027.2). The possibility that TopBP1 interacts with geminin opens a new window for elucidating unexpected but plausible roles of geminin.

4.3. Geminin-TopBP1 interaction

DNA replication and DNA repair are very closely related events and occasionally are coupled with each other. The activation of DNA repair machinery following a geminin-mediated inhibition of DNA replication can be easily imagined; however, the precise molecular target of geminin in the regulation of DNA repair remains uncertain.

TopBP1 was first identified using yeast two-hybrid screening and was shown to interact with the C-terminal region of DNA topo II beta (78). TopBP1 contains eight repeating BRCT regions, the carboxyl-terminus of BRCA1 (breast cancer susceptibility gene 1 product), throughout its sequence; it is similar to the *Saccharomyces cerevisiae* Dpb11 and *Schizosaccharomyces pombe* Cut5 checkpoint proteins and is closely related to *Drosophila* Mus101 (78,79). Using a Pfam database search, we identified seven BRCT domains (the eighth BRCT domain was found using other databases) and found the geminin interacting region to be located within the 106 ~ 295 amino acid residues of the TopBP1 protein (Figure 2). The BRCT region is a ubiquitous region homologous to regions involved in cell cycle checkpoints and DNA repair, and the BRCT regions of TopBP1 and BRCA1 bind to DNA breaks (80). The inhibition of DNA synthesis leads to the relocalization of TopBP1, together with BRCA1, to replication forks, suggesting a role in the rescue of stalled forks and also implicating TopBP1 in replication and checkpoint functions (81,82). Cell cycle checkpoints are essential for maintaining genomic integrity. TopBP1 plays a critical role in the maintenance of genomic stability during normal S phase as well as following genotoxic stress (83,84). This evidence supports a functional linkage between geminin and TopBP1 that ensures genome stability and prevents cancer formation. BRCT domains are considered as evolutionarily convenient interaction modules (85,86).

The E2F transcription factor integrates cellular signals and coordinates cell cycle progression. TopBP1 regulates E2F1 during DNA damage by recruiting E2F1 to a BRCA1-containing repair complex (87). TopBP1 is known to recruit Brg1/Brm to E2F1-responsive promoters to repress the transcriptional activity of E2F1 (88). This action could suppress E2F1-dependent apoptosis during S phase and DNA damage. Interestingly, TopBP1 is induced by E2F1 and then interacts with E2F1, indicating that E2F1 and TopBP1 form a negative feedback loop to prevent apoptosis during DNA replication (88,89).

TopBP1 has a transcriptional activation domain and two adjacent repressor domains; these repressors actively recruit repressor complexes and several chromatin modification domains (90). Indeed, TopBP1 repressed the expression of c-Abl through a novel mechanism that involved histone deacetylation and DNA methylation (91). Akt-dependent oligomerization is crucial for TopBP1 to interact with and repress E2F1 (92). Akt phosphorylation is also required for the interaction between TopBP1 and

Miz1, which is required for DNA-damage-induced cell-

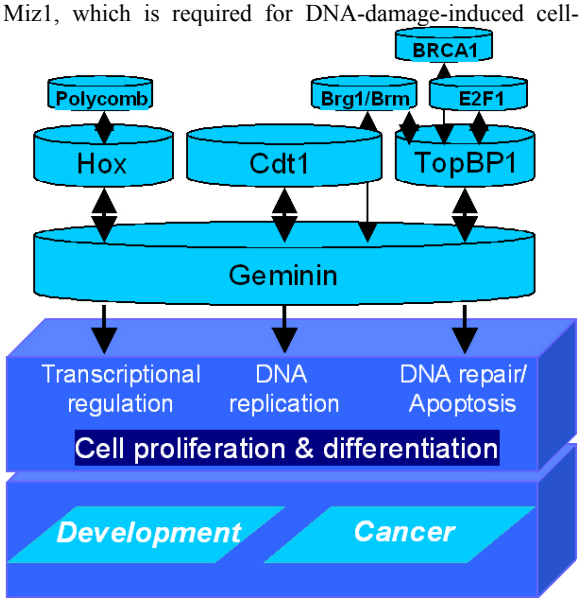

Figure 3. Geminin plays a central integrative role in multiple cellular events including cell proliferation and differentiation. The two-headed arrows with a solid line indicate protein-protein interactions. The two-headed arrows with a dotted line indicate putative protein-protein interactions revealed by yeast two-hybrid screening.

cycle arrest or human papillomavirus (HPV) transcription/replication factor E2 and the repression of Miz1 transcriptional activity. These studies define a general role for TopBP1 oligomerization in the control of transcription factors.

TopBP1 physically interacts with ATR, a key regulator of checkpoint responses to incompletely replicated and damaged DNA, and activates ATR (93,94). Geminin-deficient *Xenopus* embryos show G2 arrest of the cell cycle immediately after the midblastula transition, and the arrest requires Chk1, the effector kinase of the DNA replication/DNA damage checkpoint pathway (95). To date, several proteins have been reported to interact with TopBP1 within the framework of DNA repair and checkpoints. HECT-domain ubiquitin ligase hHYD was identified as an interactor protein of TopBP1 using yeast two-hybrid screening and was coordinated with TopBP1 during the DNA damage response (96). TopBP1 can also bind with HPV E2 and enhance the ability of E2 to activate transcription and replication (97). Promyelocytic leukemia protein colocalizes and associates with TopBP1 in response to ionizing radiation on stalled DNA loci (98).

Mutations in the BRCA genes produce increased susceptibility to breast cancer in certain families. BRCA1-IRIS was identified as a BRCA1 gene product encoded by an uninterrupted open reading frame and was co-immunoprecipitated with DNA replication licensing proteins and known replication initiation sites. The suppression of BRCA1-IRIS expression hindered the normal departure of geminin from prereplication complexes (99). But the functional interaction between BRCA1-IRIS requires further clarification. The loss of geminin causes rereplication that activates a G2/M checkpoint in human cancer cells. The ATR- and BRCA1-mediated Fanconi anemia pathway is required for the activation of a G2/M checkpoint and for DNA damage repair in response to an endogenous signal for rereplication (100). TopBP1 is now considered to be a plausible susceptibility gene for hereditary breast and/or ovarian cancer (101).

Geminin becomes phosphorylated as S phase proceeds (102). The BRCT domain interacts with its respective physiological partners in a phosphorylation-dependent manner (103). Geminin is an excellent substrate for protein kinase CK2 (104). The possibility that the geminin-TopBP1 interaction is regulated by the phosphorylation status of geminin is promising. The structure of geminin is very unique: a tetramer formed by two dimers with monomers interacting via coiled-coil domains (105). In addition to uncovering novel binding partners of geminin, the elucidation of the structural organization of geminin would provide molecular insight into its multifunctional nature. The same is true for TopBP1.

5. CONCLUDING REMARKS

The two regulatory axes of geminin-Cdt1 and geminin-homeobox proteins are conserved throughout the metazoans (106). Geminin may have evolved to maintain these axes for regulating proliferation and differentiation, and accessory systems, like geminin-TopBP1, may have evolved in metazoans to guard genome stability. As shown in Figure 3, both geminin and TopBP1 function in proximity to chromatin. Brg1/Brm is a central component of SWI/SNF complexes for regulating the status of the chromatin. Therefore, SWI/SNF complexes, including their Brg1/Brm component, may connect geminin and TopBP1. The integrity of DNA replication and gene transcription is regulated by events at the chromatin level. Similar to geminin, TopBP1 plays a role not only in DNA replication, but also in transcriptional regulation. The geminin-TopBP1 axis may be more versatile than previously imagined. Understanding the multiple roles of geminin is essential to understanding the molecular basis of the central processes in cell proliferation and differentiation.

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Abbreviations: APC/C: anaphase-promoting complex; MCM: minichromosome maintenance; CDK: cyclin-dependent kinase; topo II: topoisomerase II; TopBP1: topo II binding protein 1; EBV: Epstein Barr virus; BRCA1: breast cancer susceptibility gene 1; BRCT: BRCA1 carboxyl-terminal

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