Transcription of the *Geminin* gene is regulated by a negative-feedback loop

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ABSTRACT **Geminin performs a central function in regulating cellular proliferation and differentiation in development and also in stem cells. Of interest, down-regulation of Geminin induces gene transcription regulated by E2F, indicating that Geminin is involved in regulation of E2F-mediated transcriptional activity. Because transcription of the *Geminin* gene is reportedly regulated via an E2F-responsive region (E2F-R) located in the first intron, we first used a reporter vector to examine the effect of Geminin on E2F-mediated transcriptional regulation. We found that Geminin transfection suppressed E2F1- and E2F2-mediated transcriptional activation and also mildly suppressed such activity in synergy with E2F5, 6, and 7, suggesting that Geminin constitutes a negative-feedback loop for the *Geminin* promoter. Of interest, Geminin also suppressed nuclease accessibility, acetylation of histone H3, and trimethylation of histone H3 at lysine 4, which were induced by E2F1 overexpression, and enhanced trimethylation of histone H3 at lysine 27 and monoubiquitination of histone H2A at lysine 119 in E2F-R. However, Geminin5EQ, which does not interact with Brahma or Brg1, did not suppress accessibility to nuclease digestion or transcription but had an overall dominant-negative effect. These findings suggest that E2F-mediated activation of *Geminin* transcription is negatively regulated by Geminin through the inhibition of chromatin remodeling.**

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INTRODUCTION

Geminin is essential for development (Quinn *et al.*, 2001; Gonzalez *et al.*, 2006; Hara *et al.*, 2006) because it acts as a central regulator in governing cellular differentiation and proliferation of embryonic stem and carcinoma cells (Yang *et al.*, 2011), as well as in supporting hematopoietic stem cell activity (Ohtsubo *et al.*, 2008; Ohno *et al.*, 2010, 2013; Takihara, 2011) and mature blood cell production (Karamitros *et al.*, 2010; Shinnick *et al.*, 2010). Geminin regulates DNA replication licensing through direct interaction with Cdt1 and thus prevents rereplication (Wohlschlegel *et al.*, 2000; Tada *et al.*, 2001; Blow and Hodgson, 2002; Saxena and Dutta, 2005), and it also regulates chromatin remodeling through direct interaction with Brahma/Brg1, a catalytic subunit of the chromatin remodeling factor, SWI/SNF (Kroll *et al.*, 1998; Muchardt and Yaniv, 2001; Seo *et al.*, 2005; Yellajoshyula *et al.*, 2011, 2012). Geminin is further implicated in transcriptional regulation through direct interaction with the Polycomb-group (PcG) complex 1 (also designated as Polycomb repressive complex 1; Luo *et al.*, 2004), a subset of Hox and Six3, homeodomain transcription factors (Del Bene *et al.*, 2004; Luo *et al.*, 2004). Its expression is regulated at the protein level through the ubiquitin-proteasome system containing multiple E3 ubiquitin

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Abbreviations used: APC/C, anaphase-promoting complex/cyclosome; ChIP, chromatin immunoprecipitation; E2F-R, E2F-responsive region; H2AK119ub, mono-ubiquitination of histone H2A at lysine 119; H3ac, acetylation of histone H3; H3K4me3, tri-methylation of histone H3 at lysine 4; H3K27me3, tri-methylation of histone H3 at lysine 27; PcG, Polycomb group.

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ligases. The anaphase-promoting complex/cyclosome (APC/C) generates the oscillating expression pattern in the cell cycle (McGarry and Kirschner 1998), and the PcG complex 1 (Ohtsubo *et al.*, 2008) and the RDCOX complexes, which are composed of Roc1-Ddb1-Cul4a and Hoxa9 (Ohno *et al.*, 2013) or Hoxb4 (Ohno *et al.*, 2010), regulate expression levels of the Geminin protein. PcG complexes regulate transcription of *Hox* genes through epigenetic chromatin modification (Wang *et al.*, 2004; Takihara, 2008; Yasunaga *et al.*, 2013), indicating that the PcG complex 1 and Hox make up a molecular network regulating Geminin expression levels (Yasunaga *et al.*, 2013). Geminin expression is thus strictly regulated during mammalian development and stem cell regulation. Down-regulation of Geminin induces cellular differentiation (Yang *et al.*, 2011) and up-regulated transcription of genes under the regulation of E2F family members (Ohno *et al.*, 2010). Nine E2F family members are present in mammals: E2F1, 2, 3a, 3b (an alternative product from the *E2f3* gene; Leone *et al.*, 2000), and 4–8 (Stevens and La Thangue 2003; van den Heuvel and Dyson 2008). E2F family members form heterodimeric complexes with DP1 or DP2 and bind to DNA in a sequence-specific manner (Stevens and La Thangue, 2003). E2F1, 2, and 3a are categorized as transcriptional activators and E2F3b and 4–8 as transcriptional repressors (DeGregori *et al.*, 1997; van den Heuvel and Dyson, 2008). E2F1, 2, 3a, and 3b interact with Rb, which suppresses the transcriptional activator function and silences E2F-responsive targets so that exit from the cell cycle is facilitated (Dunaief *et al.*, 1994; Zhang *et al.*, 2000; Dahiya *et al.*, 2001). On the other hand, E2F4 and 5 may repress transcription in quiescent cells in an Rb family member–dependent manner, and E2F6–8 may do so in an Rb family–independent manner. A series of genes that regulate cell cycle, DNA replication, DNA damage response, and apoptosis is regulated by E2F family members (Stevens and La Thangue, 2003; van den Heuvel and Dyson, 2008). Geminin may thus govern cellular proliferation as well as cellular differentiation, not only through direct regulation of DNA replication and chromatin remodeling, but also through its transcriptional regulatory activity. Although direct interaction of Geminin with PcG complex 1 and a subset of Hox and Six3, homeodomain transcription factors, is implicated in transcriptional regulation (Del Bene *et al.*, 2004; Luo *et al.*, 2004), involvement of Geminin in E2F-mediated transcriptional regulation is largely unknown. We previously found that down-regulation of Geminin protein up-regulates transcription of the *Geminin* (*Gmnn*) gene (Ohno *et al.*, 2010), which suggests that Geminin expression may be regulated by a molecular feedback loop. Although the *Geminin* gene is transcriptionally regulated by E2F family members (Markey *et al.*, 2004; Yoshida and Inoue, 2004), it is unclear how Geminin is involved in E2F-mediated transcriptional regulation of the *Geminin* gene. In this study we examine the effect of Geminin on the transcriptional activity of the *Geminin* promoter, as well as on chromatin configuration.

RESULTS

We first performed a luciferase reporter assay of NIH 3T3 cells to examine the effect of Geminin on E2F-mediated transcriptional activation of the *Geminin* gene promoter. The luciferase reporter vector driven by the human *Geminin* (*GMNN*) promoter including E2F-R in the first intron (Figure 1; Yoshida and Inoue, 2004) was transiently cotransfected with each of the expression vectors for representative human E2F family members, that is, hemagglutinin (HA)-tagged E2F1, 2, 3a, and 4–7 (Yoshida and Inoue, 2004). We did not examine E2F3b at this position because the functions of E2F3a and E2F3b reportedly largely overlap, and E2F3a, but not E2F3b, deficiency was found to cause significant proliferation defects

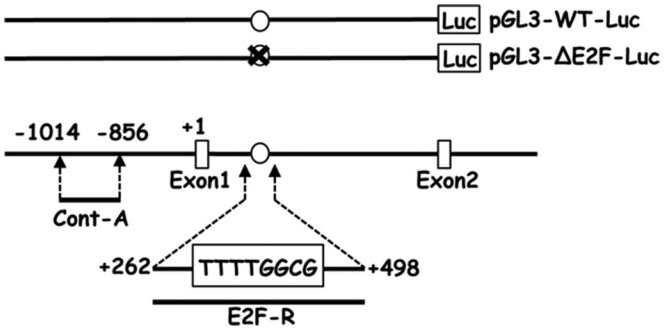

FIGURE 1: Structure of the luciferase reporter for the *Geminin* gene promoter. E2F-R in the first intron of the mouse *Geminin* gene is indicated by a white circle. pGL3-WT-Luc, the luciferase reporter for the human *Geminin* gene promoter, possesses the region from −2736 to +2244 base pairs, while pGL3-ΔE2F-Luc possesses the same region with the mutation in E2F-R, which is indicated by X. For the ChIP and nuclease accessibility assays, E2F-R (from +262 to +498 base pairs) and one control region, Cont-A (from −1014 to −856 base pairs), were detected by PCR analysis in Figures 5 and 6.

(Danielian *et al.*, 2008). Luciferase activity was examined 48 h after the transfection. Overexpression of HA-E2F1, 2, and 3a was seen to induce reporter activity for the *Geminin* promoter depending on E2F-R (Figure 2A), whereas cotransfection of Geminin suppressed the reporter activity induced by HA-E2F1 and HA-E2F2 overexpression but did not suppress that induced by HA-E2F3a overexpression (Figure 2A). HA-E2F5–7 overexpression tended to have a mildly repressive effect on the reporter activity, giving rise to down-regulation of the luciferase activity at a level similar to that in the luciferase reporter vector with mutations in E2F-R (Figure 2A). Moreover, Geminin cotransfection mildly synergized with the repressive effect of HA-E2F5–7 on the reporter activity, which in part depended on E2F-R of the reporter gene (Figure 2A). No distinct effect of Geminin cotransfection on HA-E2F4 was observed (Figure 2A). With a series of reporter assays, we were able to confirm by immunoblot analysis that transfectants caused overexpression of each of the transfected HA-E2F family members, as well as of Geminin and Geminin5EQ (Figure 2A). In addition, we confirmed that exogenous Geminin expression did not affect expression of HA-E2F family members and that expression levels of E2F family members, Geminin, and Geminin5EQ were comparable among immunoblot analyses shown in Figure 2, A and B (Supplemental Figure S1). We also confirmed by means of cell cycle analysis with bromodeoxyuridine (BrdU) that cell cycle status was not significantly affected by transfection in this particular assay (Supplemental Figure S2A). Although Geminin was thus shown to suppress E2F1-mediated transcriptional activation, immunoprecipitation analyses with an anti-Rb antibody did not show a direct association of HA-E2F1 or Rb with Geminin conjugated with Flag tag (Flag-Geminin) in HEK-293 cells (Figure 3) in the condition that direct interaction of Myc-Cdt1 with Flag-Geminin was detectable (Figure 3). The same was true for an anti-HA antibody (Figure 3), suggesting that the effect of Geminin overexpression on E2F1-mediated transcriptional activation does not directly affect E2F1 or RB.

Although transiently transfected DNA is subjected to chromatin assembly, the efficiency may vary, and the assembled chromatin may not completely reflect the physiological chromatin context (Wolffe, 1998). In addition, the nucleotide sequence in E2F-R is highly conserved between human and mouse (Yoshida and Inoue, 2004). We therefore examined the effect of E2F1 and Geminin

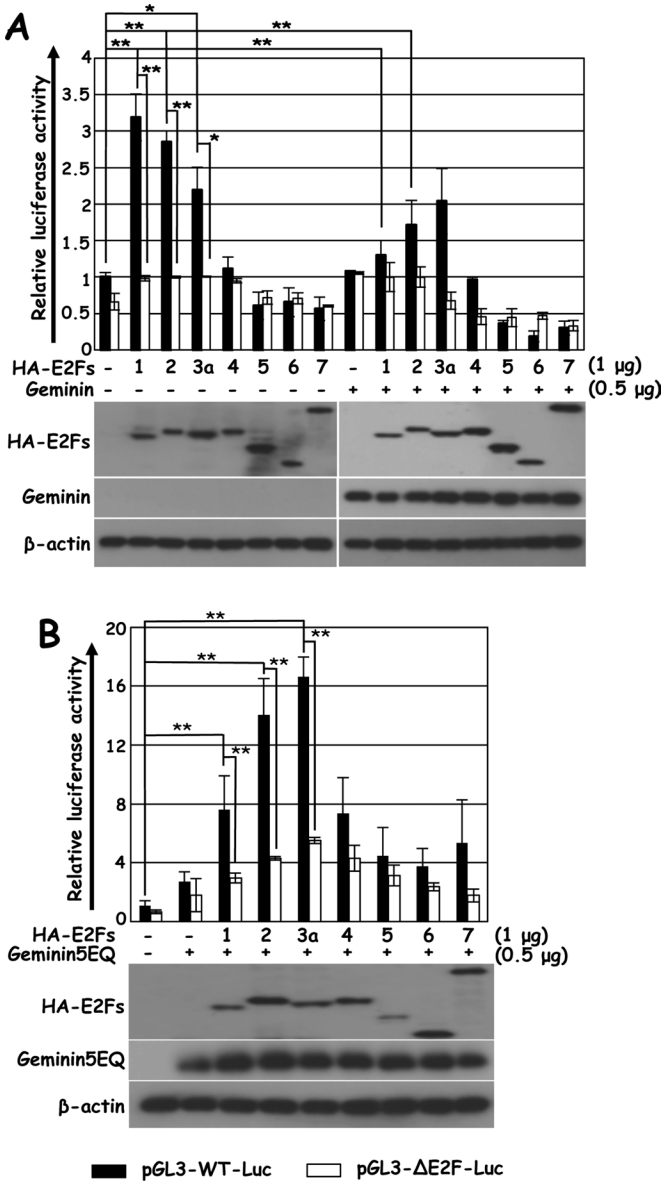

FIGURE 2: Effect of Geminin on E2F-mediated transcriptional activation. A transient transfection assay with a luciferase reporter was performed for the *Geminin* promoter. Top, relative luciferase activity. Bottom, the immunoblot analysis. HA-E2F family members (HA-E2Fs) and Geminin were detected by means of immunoblot analysis using anti-HA and anti-Geminin antibodies, respectively. Black bars, pGL3-WT-Luc; white bars, pGL3-ΔE2F-Luc. (A) Relative luciferase activity in cotransfectants with HA-E2Fs and wild-type Geminin. (B) Relative luciferase activity in cotransfectants with HA-E2Fs and Geminin5EQ. Statistical significance is as follows: $*P < 0.1$; $**P < 0.05$; $***P < 0.01$.

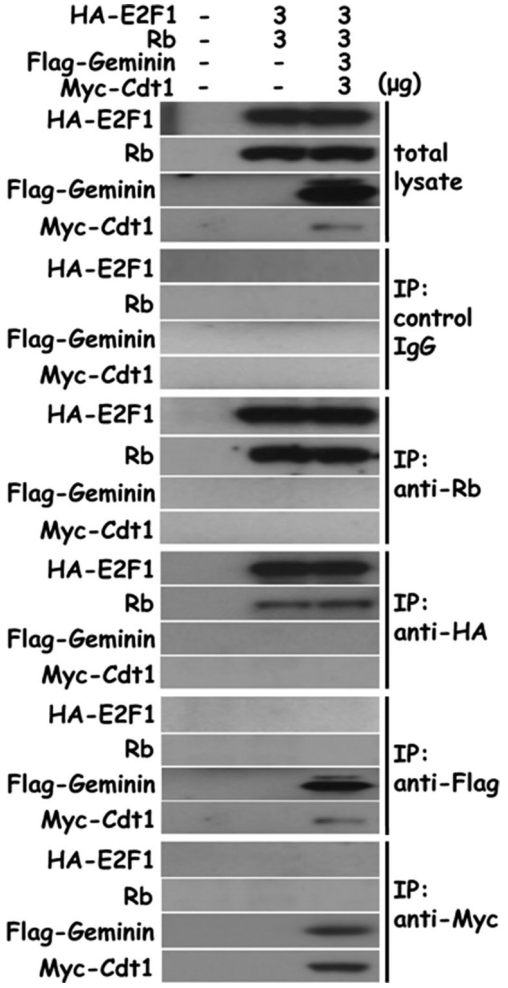

FIGURE 3: Immunoprecipitation analysis of E2F1, Rb, and Geminin. HEK-293 cells were transiently transfected with HA-E2F1, Rb, and 6Myc-Geminin, and the immunoprecipitates obtained with either a polyclonal anti-Rb or an anti-HA antibody were examined by immunoblot analysis. HA-E2F1, Rb, and 6Myc-Geminin were examined with the aid of, respectively, monoclonal anti-HA, anti-Rb, and anti-Myc antibodies. Direct interaction of HA-E2F1 with Rb was detected, as described previously (van den Heuvel and Dyson 2008), but not that of Geminin with either HA-E2F1 or Rb.

transfection on transcription of the endogenous genomic *Geminin* gene in NIH 3T3 cells to facilitate determination of the effect of E2F1 and Geminin on chromatin configuration (Figure 4) because E2F1 and Geminin were reportedly involved in epigenetic transcriptional regulation (Takahashi *et al.*, 2000; Tyagi *et al.*, 2007) and chromatin remodeling (Seo *et al.*, 2005), respectively. In this study we prepared an expression vector for *Geminin* cDNA that deleted a majority of the 3′–untranslated region (UTR) and used a real-time reverse transcription (RT)-PCR system for detecting the 3′-UTR, which is able to distinguish endogenous from exogenous *Geminin* mRNA. We first confirmed that overexpression of exogenous 6Myc-tagged (6Myc-) Geminin suppressed HA-E2F1–mediated transcriptional activation of the *Geminin* gene in NIH 3T3 cells in a dose-dependent manner (Figure 4). We confirmed that overexpression of HA-E2F1 and/or 6Myc-Geminin did not significantly alter cell cycle status in this assay condition (Supplemental Figure S3).

We further examined whether Geminin-mediated transcriptional repression depends on transcriptional activation induced by E2F family members. Although luciferase reporter assay of NIH 3T3 cells showed that Geminin efficiently suppressed transcriptional activity induced by *E2F1* and *E2F2*, *E2F2* mRNA expression was much lower than *E2F1* mRNA in NIH 3T3 cells (Figure 5A). Then we performed small interfering RNA (siRNA)–mediated knockdown of E2F1. Knockdown of E2F1 abrogated transcriptional repression effect of Geminin and the transcriptional augmentation effect of Geminin5EQ on the *Geminin* gene, whereas supertransfection of HA-E2F1 recovered the transcriptional repression and augmentation activities of 6Myc-Geminin and Flag-Geminin5EQ, respectively, indicating that

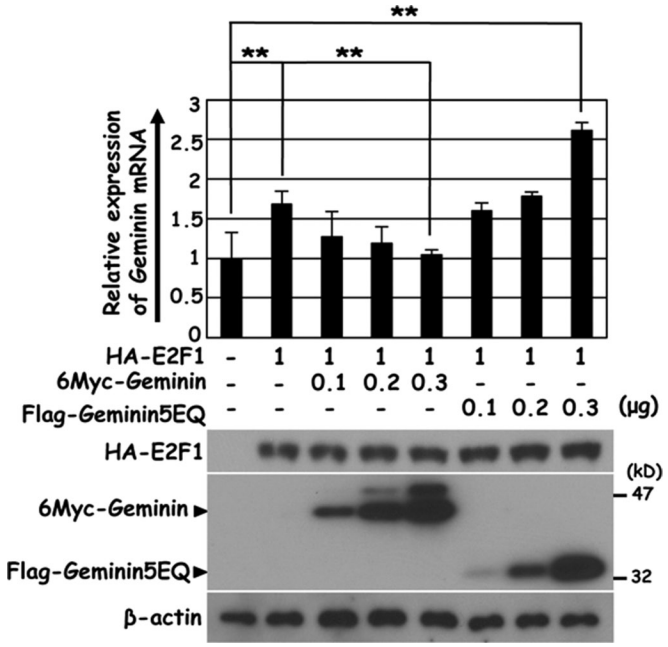

FIGURE 4: Effect of Geminin on E2F1-mediated transcriptional activation of the *Geminin* gene. NIH 3T3 cells were cotransfected with HA-E2F1, 6Myc-Geminin, or Flag-Geminin5EQ, and their effect on expression of the *Geminin* gene was examined by means of real-time reverse transcription-PCR analysis, which exclusively detects endogenous mRNA for the *Geminin* gene (top). For immunoblot analysis of the transfectants (bottom), HA-E2F1, 6Myc-Geminin, and Flag-Geminin5EQ were detected by anti-HA and anti-Geminin antibodies, respectively. Statistical significance is as follows: $^{**}P < 0.05$.

Geminin specifically regulates E2F1-mediated transcriptional activation in NIH 3T3 cells (Figure 5B). Here knockdown of E2F1 and supertransfection of 6Myc-Geminin, Flag-Geminin5EQ, or HA-E2F did not significantly alter cell cycle status in this assay condition, and knockdown of Geminin, described layer, also did not (Supplemental Figure S4).

To examine the effect of E2F1 and Geminin overexpression on chromatin configuration in the *Geminin* promoter region, we next performed chromatin immunoprecipitation (ChIP) analysis (Figure 6). Exogenously transfected HA-E2F1, clearly associated with E2F-R and overexpression of Geminin, reduced this association as detected by ChIP analysis with an anti-HA antibody (Figure 6A). However, we could not detect any changes in Geminin, Brahma, and Brg1 binding to E2F-R as a result of overexpression of HA-E2F1 and/or Flag-Geminin (Figure 6A), nor could we detect any significant regional preferences of Geminin, Brahma, and Brg1 binding in the chromatin region examined (Figure 6A). We also examined histone modifications in E2F-R of the *Geminin* gene. Increased acetylation of histone H3 (H3ac) and trimethylation of histone H3 at lysine 4 (H3K4me3) were observed in HA-E2F1–transfected NIH 3T3 cells, which, surprisingly, were suppressed by Flag-Geminin cotransfection (Figure 6B). Trimethylation of histone H3 at lysine 27 (H3K27me3), as well as monoubiquitination of histone H2A at lysine 119 (H2AK119ub), was enhanced by Flag-Geminin cotransfection (Figure 6B). On the other hand, no significant changes in binding of representative members of PcG complex 1 (Ring1B(Rnf2), Bmi1, Rae28(Phc1), and Scmh1) was not detected in any of the transfectants (Figure 6C), although Geminin was previously shown to form a complex with PcG complex 1 (Luo *et al.*, 2004). The altered H2AK119ub tended to be present even in the Cont-A region of all

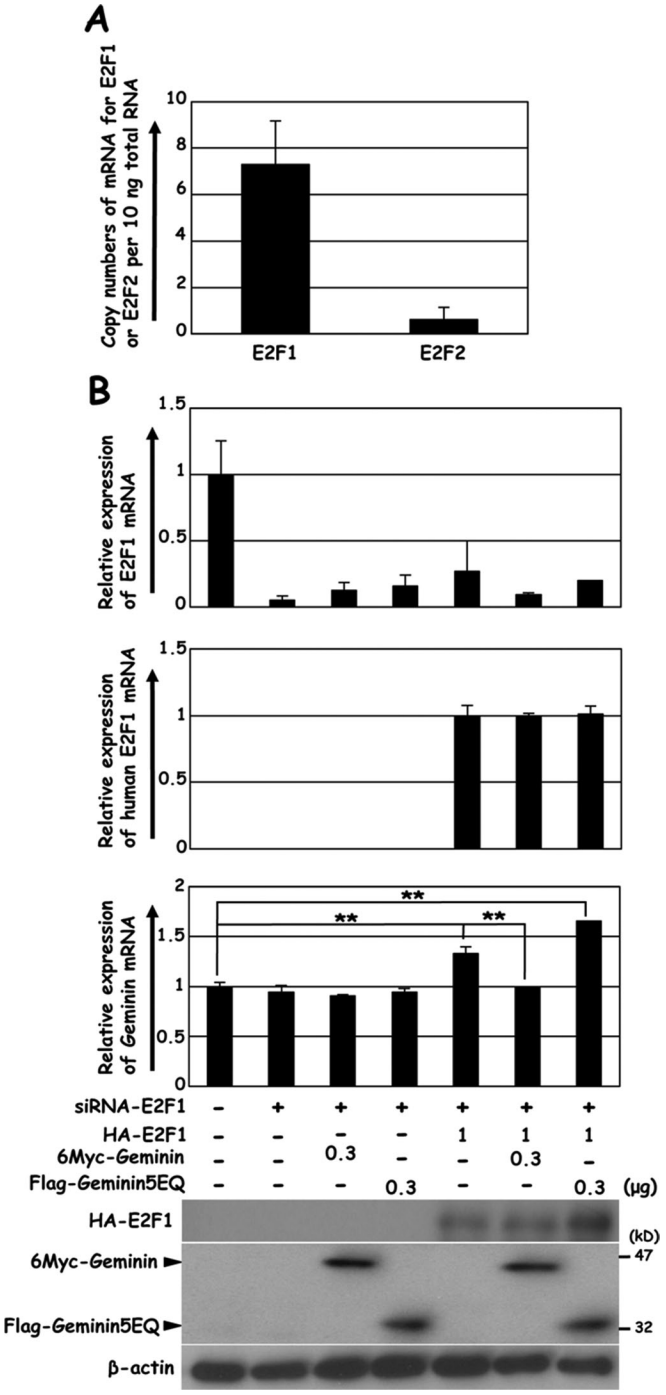

FIGURE 5: Effect of siRNA-mediated E2F1 knockdown on transcriptional repression by Geminin. (A) Expression of *E2F1* and *E2F2* mRNA in NIH 3T3 cells. mRNA for *E2F1* and *E2F2* was examined by real-time reverse transcription-PCR analysis. (B) Effect of siRNA-mediated knockdown of E2F1 on transcription of the *Geminin* gene. siRNA-mediated down-regulation of mRNA for *E2F1* and exogenous expression of mRNA for HA-E2F1 were confirmed by real-time reverse transcription-PCR analysis. mRNA from the endogenous *Geminin* gene was examined by using a real-time reverse transcription-PCR system for detecting the 3′-UTR. Statistical significance is as follows: $^{**}P < 0.05$.

the transfectants. Using control immunoglobulin G (IgG) and an anti–histone H3 antibody as control antibodies, we detected very few immunoprecipitates with control IgG, which showed no

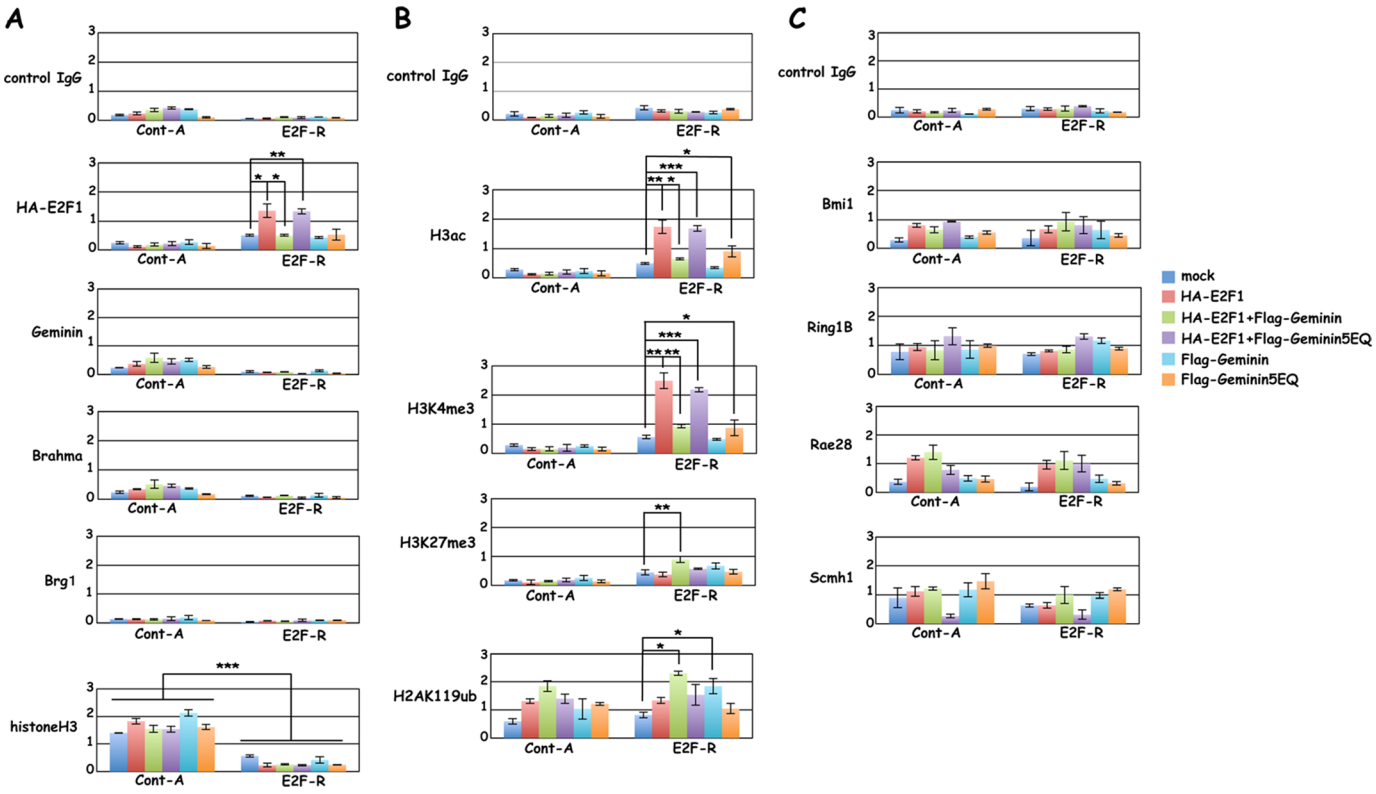

FIGURE 6: ChIP analysis of the promoter region of the *Geminin* gene. NIH 3T3 cells were transiently cotransfected with HA-E2F1 (1 μg), Flag-Geminin, or Flag-Geminin5EQ (0.3 μg) and subjected to ChIP analysis of the *Geminin* gene with the following antibodies: (A) anti-HA, anti-Geminin, anti-Brahma, anti-Brg1, anti–histone H3, (B) anti-H3ac, anti-H3K4me3, anti-H3K27me3, and anti-H2AK119ub, or (C) a series of antibodies against representative members of PcG complex 1. Statistical significance is as follows: $*P < 0.1$; $**P < 0.05$; $***P < 0.01$.

significant differences between Cont-A and E2F-R (Figure 6A). With the anti–histone H3 antibody, on the other hand, we detected a much weaker signal in the E2F-R than in the Cont-A region in all the transfectants (Figure 6A), which may reflect an open chromatin configuration in E2F-R. Because Geminin reportedly inhibits the activity of the chromatin-remodeling complex through direct binding with Brahma and Brg1 (Seo *et al.*, 2005), we examined the accessibility of chromatins to nuclease digestion by in situ chromatin digestion with a nuclease (Yellajoshyula *et al.*, 2011) and found that HA-E2F1 transfection induced such accessibility of E2F-R of the *Geminin* gene (Figure 7). In contrast, additional cotransfection of wild-type 6Myc-Geminin suppressed the nuclease accessibility induced by HA-E2F1 transfection (Figure 7).

We next attempted to specifically eliminate the molecular function of Geminin in the inhibition of chromatin remodeling by using the Geminin5EQ mutant, in which glutamine (Q) and asparagine (N) were substituted for, respectively, glutamic (E) and aspartic (D) acids in the Geminin-Brahma/Brg1 interaction domain, and this reportedly eliminates the physical interaction with Brahma and Brg1 (Seo *et al.*, 2005). The molecular interaction of Flag-tagged domain II (amino acids [aa] 314–570) of Brahma (Flag-Brahma-DomII) or Flag-tagged domain II (aa 342–598) of Brg1 (Flag-Brg1-DomII) with wild-type 6Myc-Geminin was detected by means of immunoprecipitation analysis, whereas that of HA-Geminin5EQ with Flag-Brahma-DomII or Flag-Brg1-DomII was not (Figure 8, A and B), as reported previously (Seo *et al.*, 2005). Because Geminin was previously reported to form a dimer (Benjamin *et al.*, 2004; Lee *et al.*, 2004; Saxena *et al.*, 2004) or a multimer (Lutzmann *et al.*, 2006), we used immunoprecipitation analysis for further examination of the molecular interaction of 6Myc-Geminin and Flag-Geminin5EQ in HEK-293 cells and found evidence of such interaction (Figure 8, A and B). Furthermore, surprisingly, overexpression of HA-Geminin5EQ

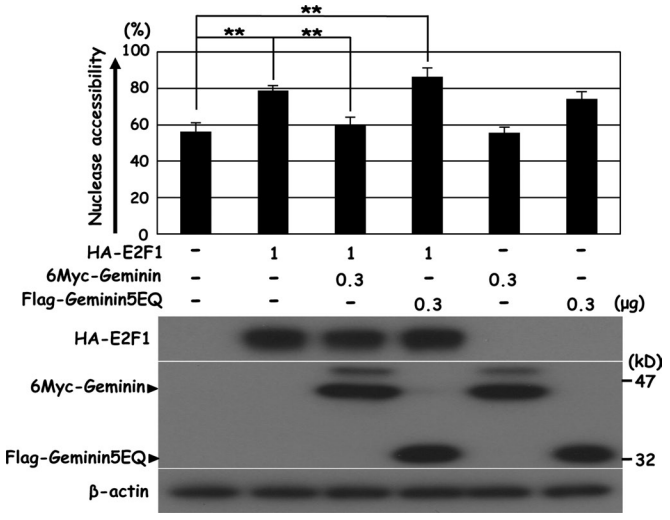

FIGURE 7: Effect of Geminin on E2F1-mediated augmentation of nuclease accessibility. NIH 3T3 cells were transiently cotransfected with HA-E2F1 and 6Myc-Geminin or Flag-Geminin5EQ and subjected to a nuclease accessibility assay (top) and immunoblot analyses of the transfectants with anti-HA and anti-Geminin antibodies (bottom). The susceptibility of E2F-R in the *Geminin* gene to nuclease digestion is shown as nuclease accessibility. Statistical significance is as follows: $*P < 0.05$.

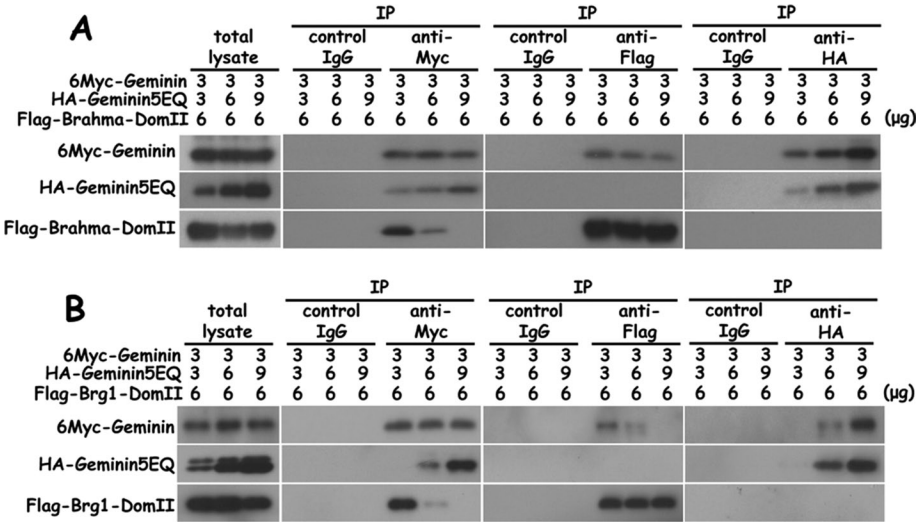

FIGURE 8: Immunoprecipitation analysis of molecular interaction of Geminin with Brahma or Brg1 in HEK-293 cells. Effect of HA-Geminin5EQ on molecular interaction of 6Myc-Geminin with Flag-Brahma-DomII (A) or Flag-Brg1-DomII (B). Note that HA-Geminin5EQ bound 6Myc-Geminin and eliminated molecular interaction of 6Myc-Geminin with Flag-Brahma-DomII or Flag-Brg1-DomII.

abrogated the molecular interaction of 6Myc-Geminin with Flag-Brahma-DomII or Flag-Brg1-DomII in a dose-dependent manner, suggesting that Geminin5EQ overexpression prevents the molecular interaction of wild-type Geminin with Brahma/Brg1 in a dominant-negative manner (Figure 8, A and B). In contrast to that of wild-type Geminin, overexpression of Geminin5EQ did not suppress the reporter activity but, instead, markedly augmented the activity induced by overexpression of HA-E2F1 and HA-E2F2 (Figure 2B), probably through its dominant-negative effect on the molecular interaction of the endogenous wild-type Geminin molecule with Brahma/Brg1, and Geminin5EQ showed the augmented reporter activity synergistically with E2F3a-7 (Figure 2B). We confirmed that overexpression of HA-E2F and/or Geminin5EQ did not significantly alter cell cycle status (Supplemental Figure S2B). Furthermore, H3ac and H3K4me3 remained elevated, whereas H3K27me3 and H2AK119ub did not increase in E2F-R of transfectants with HA-E2F1 and HA-Geminin5EQ (Figure 6B). Finally, a moderate increase in H3ac and H3K4me3 was detected in transfectants with HA-Geminin5EQ only (Figure 6B), and nuclease accessibility in E2F-R was augmented in the Flag-Geminin5EQ transfectants (Figure 7). These findings indicate that HA-E2F1 or HA-E2F2 overexpression activates transcription of the *Geminin* gene through the induction of chromatin accessibility and histone modification, but that Geminin overexpression reduces chromatin accessibility and altered histone modification induced by E2F1 overexpression through the inhibitory effect on chromatin remodeling, which may result in suppression of transcriptional activation.

We further examined effect of overexpression of Geminin on E2F-mediated transcription activation of the *Cyclin A2* (*Ccna2*) and *Mcm7* genes. These genes are known to be regulated by members of the E2F family (Schulze *et al.*, 1995; Takahashi *et al.*, 2000; Yoshida and Inoue, 2004). Although expression of the *β-actin* (*Actb*) gene, an E2F-unresponsive control gene, was not affected by either transfection with HA-E2F1 or cotransfection with HA-E2F1 combined with 6Myc-Geminin or Flag-Geminin5EQ (Figure 9C), transfection with 6Myc-Geminin suppressed HA-E2F1–mediated transcription activation of the *Cyclin A2* and *Mcm7* genes (Figure 9, A and B). In contrast, transfection of Flag-Geminin5EQ did not suppress but augmented the E2F1-mediated transcriptional activation in the *Cyclin A2* gene (Figure 9, A and B). We also examined effect of Geminin knockdown on transcription of the *Cyclin A2* and *Mcm7* genes. In contrast to Geminin overexpression, Geminin knockdown augmented transcription of these genes, which was suppressed by supertransfection of 6Myc-Geminin and further augmented by supertransfection of Flag-Geminin5EQ (Figure 10). These findings were in good agreement with those obtained with observation of the effect of Geminin on E2F1-mediated transcriptional activation of the *Geminin* gene. This suggests that Geminin may, as a rule, suppress E2F1- or E2F2-mediated transcription activation via its inhibitory effect on chromatin remodeling, which is consistent with the negative-feedback effect of Geminin on transcription of the *Geminin* gene as described here.

DISCUSSION

Geminin suppressed E2F1- and E2F2-mediated, but not E2F3a-mediated, transcriptional activation of the *Geminin* promoter in the transient transfection assay of NIH 3T3 cells. On the other hand, Geminin had a moderate synergistic effect on the transcriptional repression by E2F5–7, which partially depended on the presence of E2F-R. A similar suppressive effect of Geminin on E2F1-mediated transcriptional activation was observed in the *Geminin* gene in a dose-dependent manner. No direct binding of Geminin with E2F1 or with Rb was observed, however, which suggests that Geminin may be involved in transcriptional regulation through higher-order chromatin regulation. Epigenetic histone modification was compatible with transcriptional induction by E2F1 overexpression and transcriptional repression by Geminin overexpression (Suganuma and Workman, 2008), that is, H3ac and H3K4me3 were induced by E2F1 and suppressed by Geminin overexpression. By the same token, H3K27me3 and H2AK119ub were reduced by E2F1 overexpression but increased by Geminin overexpression. Binding of components of PcG complex 1, that is, Ring1B, Bmi1, Rae28, and Scmh1 (Ohtsubo *et al.*, 2008), in E2F-R of the *Geminin* gene was not greatly affected by overexpression of either E2F1 or Geminin. An important finding, however, is that nuclease accessibility was enhanced by E2F1 overexpression but diminished by Geminin overexpression. This suggests that Geminin affects E2F1-mediated transcriptional activation via regulation of epigenetic histone modification and chromatin configuration (Figures 5 and 6) and this may be a molecular mechanism that can explain how down-regulation of Geminin up-regulated a series of E2F-responsive genes in our previous study (Ohno *et al.*, 2010). In addition, since we could demonstrate that Geminin, in combination with E2F5–7, exerts a synergistic repressive effect, it may be also involved in transcriptional activation of E2F-responsive genes resulting from down-regulation of Geminin. Although it is known that transient reporter assays may not completely reflect the effect of chromatin regulation (Wolffe, 1998), in our study the effect of Geminin overexpression on the reporter assay correlated well with that on the genomic *Geminin* gene. Because Geminin was shown to inhibit chromatin remodeling

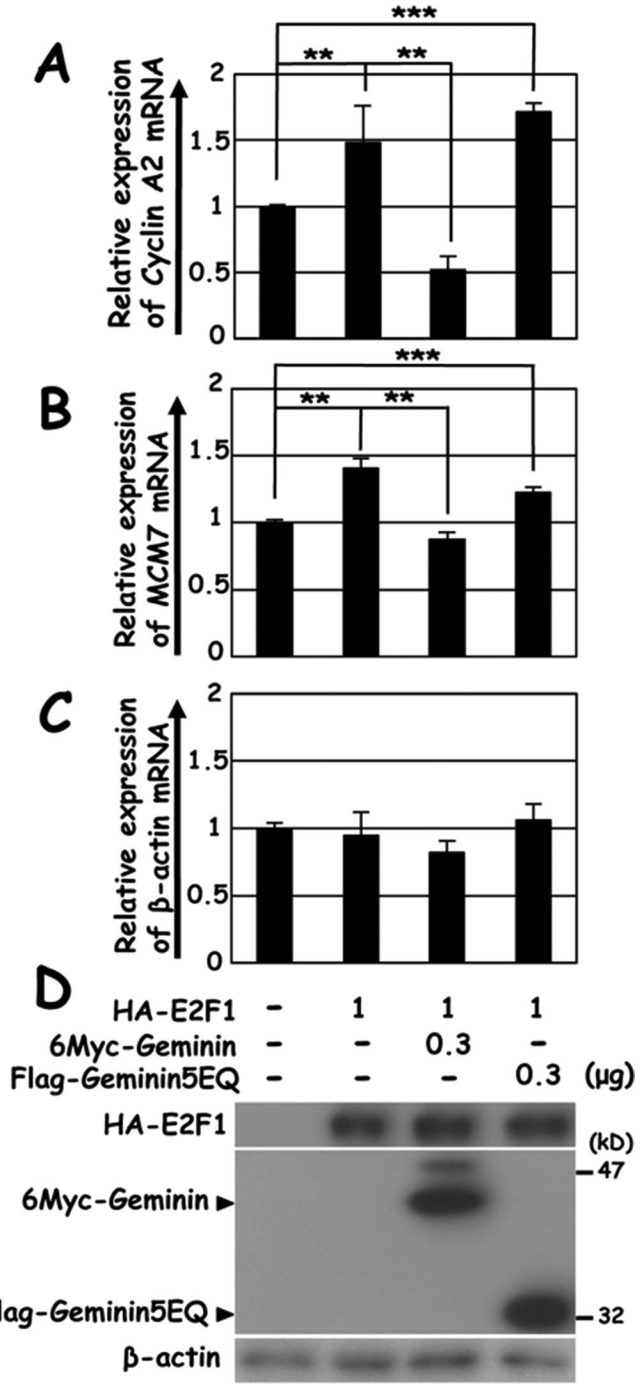

FIGURE 9: Effect of Geminin on E2F1-mediated transcriptional activation of the *Cyclin A2* and *Mcm7* genes. NIH 3T3 cells were transiently transfected with HA-E2F1, and the effect of 6Myc-Geminin or Flag-Geminin5EQ supertransfection on mRNA expression from the genomic *Cyclin A2* and *Mcm7* genes was examined by real-time reverse transcription-PCR analysis. Relative expression levels of mRNA were calculated by normalizing them with the expression level of *Gapdh* mRNA. The effect on the *β-actin* gene was examined as an E2F1-unresponsive control. Relative expression of mRNA: (A) *Cyclin A2*, (B) *Mcm7*, and (C) *β-actin*. (D) Immunoblot analyses of the transfectants with anti-HA and anti-Geminin antibodies. Statistical significance is as follows: $^{**}P < 0.05$; $^{***}P < 0.01$.

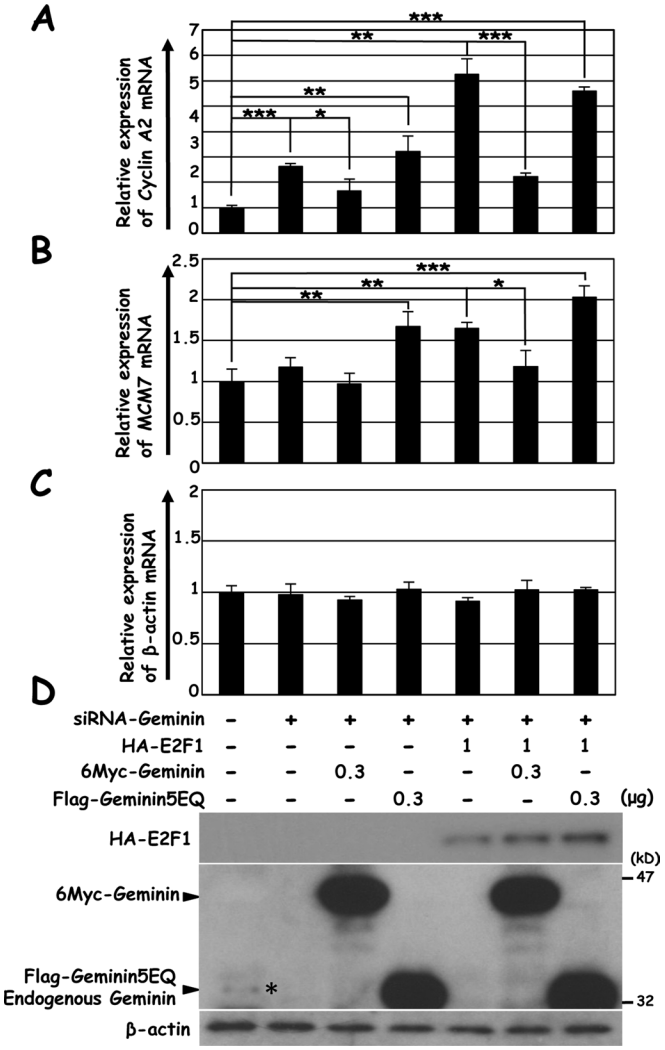

FIGURE 10: Effect of Geminin knockdown on E2F1-mediated transcriptional activation of the *Cyclin A2* and *Mcm7* genes. Geminin expression was knocked down by transfection of the siRNA, and the effect on mRNA expression from the genomic *Cyclin A2* and *Mcm7* genes was examined in the presence or absence of HA-E2F1 transfection. The effect of supertransfection of 6Myc-Geminin or Flag-Geminin5EQ was also examined. Relative expression levels of mRNA were calculated by normalizing them with the expression level of *Gapdh* mRNA. The effect on the β-*actin* gene was examined as an E2F1-unresponsive control. Relative expression of mRNA: (A) *Cyclin A2*, (B) *Mcm7*, and (C) β-*actin* (D). Immunoblot analyses of the transfectants with anti-HA and anti-Geminin antibodies. Asterisk indicates endogenous Geminin. Statistical significance is as follows: $^{*}P < 0.1$; $^{**}P < 0.05$; $^{***}P < 0.01$.

through direct inhibition of the chromatin remodeling factors Brahma and Brg1 (Seo *et al.*, 2005), we applied the Geminin5EQ mutant, which does not interact with Brahma and Brg1 (Seo *et al.*, 2005), and attempted to scrutinize the molecular mechanism of how Geminin overexpression reduces nuclease accessibility and transcriptional activity. Of interest, Geminin5EQ neither suppressed transcriptional activation nor reduced nuclease accessibility induced by E2F1 overexpression. Because Geminin5EQ does not exert the effect of wild-type Geminin on transcriptional repression or its negative effect on nuclease accessibility, the inhibitory effect of Geminin on chromatin remodeling may be essential for Geminin to exert its transcriptional repressive effect. Geminin is thus presumed to affect E2F1-induced transcriptional activation through direct regulation of chromatin remodeling. This may be one reason why good correlation was observed between the findings for the

effect of Geminin overexpression on *Geminin* transcription by transiently transfected reporter DNA and the genomic *Geminin* gene promoter (Narlikar *et al.*, 2002). Here we showed that Geminin clearly exerted a transcriptional repressive effect on E2F1-mediated transcriptional activation in NIH 3T3 cells. The present evidence, on the other hand, indicates that the effect of Geminin on transcription regulation is mediated by the inhibitory effect on Brahma/Brg1. Thus the effect of Geminin on transcriptional regulation may not be limited to E2F1-mediated transcriptional activation, which was shown in NIH 3T3 cells, but may further cover transcriptional regulatory events in which chromatin-remodeling complexes including Brahma/Brg1 are involved. Although previous studies reported that Geminin maintained hyperacetylated and accessible chromatin configurations, as well as a bivalent epigenetic state in neural fate-promoting genes (Yellajoshyula *et al.*, 2011, 2012), the inhibitory effect of Geminin on chromatin remodeling may be involved in inducing either accessibility or inaccessibility of chromatin configuration, and either transcriptional activation or repression depends on chromatin loci or cell contexts, as previously suggested (Narlikar *et al.*, 2002; Nagl *et al.*, 2007). Further analyses may be required to deepen understanding of a role for Geminin in transcriptional regulation and confirm implication of the findings in more physiological aspects because we performed all the experiments here by using the transient transfection assay in vitro.

Geminin expression is regulated at the transcriptional level, as well as at the protein level. Transcription of the *Geminin* gene is regulated by E2F family members, whereas Geminin protein is under the regulation of the ubiquitin-proteasome system with APC/C, PcG complex 1, and RDCOX complexes as E3 ubiquitin ligase. We previously proposed that, since *Hox* genes are epigenetically regulated by PcG complex 1, PcG complex 1 and a subset of the downstream *Hox* genes may form a regulatory network for tuning the expression level of Geminin protein (Yasunaga *et al.*, 2013). In the study presented here, we provide evidence that transcription of the *Geminin* gene is regulated by a negative-feedback loop, further strengthening our hypothesis that Geminin expression level is homeostatically tuned by a regulatory network. The present study may help clarify how exactly Geminin expression levels are governed to couple regulation of transcription with DNA replication for stem cell regulation and development. Furthermore, E2F family member–mediated transcriptional activation of genes, such as *Cyclin A2* and *Mcm7*, was also suppressed by Geminin but not by Geminin5EQ, indicating that Geminin suppresses E2F-mediated transcriptional activation via its inhibitory effect on chromatin remodeling. As a result of this effect, regulation of the genes by E2F family members may thus be transcriptionally regulated by Geminin. In turn, this may imply a molecular role for Geminin in transcriptional regulation, although Geminin is known to regulate transcription through direct interaction with the homeodomains of a subset of Hox proteins (Luo *et al.*, 2004), six3 (Del Bene *et al.*, 2004), and the PcG complex 1 (Luo *et al.*, 2004). Although Geminin inhibits cellular proliferation and differentiation through the negative regulation of, respectively, Cdt1 and the chromatin-remodeling complex, the transcriptional regulatory effect of Geminin via chromatin remodeling may be involved not only in cellular differentiation but also in cellular proliferation, because the majority of genes involved in cell cycle regulation and DNA replication are regulated by E2F family members. Geminin may thus suppress transcription when active transcription factors are present in cells by inhibiting chromatin remodeling. Moreover, Geminin not only induces quiescence by inhibiting Cdt1, but also stabilizes Cdt1 to secure DNA replication in the next round of the cell cycle (Ballabeni *et al.*, 2004; Tsunematsu *et al.*, 2013). This leads to the hypothesis that high Geminin expression at the G_0/G_1 phase (Ohtsubo *et al.*, 2008; Yang *et al.*, 2011) provides a crucial molecular mechanism not only for inducing cell quiescence, but also for maintaining the potential for DNA replication and transcription in stem cells.

MATERIALS AND METHODS

TaqMan real-time PCR

Total cellular RNA was extracted from cells with the Quick-RNA MicroPrep Kit (ZYMO Research, Orange, CA), and was reverse transcribed using TaqMan Reverse Transcription Reagents (Life Technologies, Carlsbad, CA). The product was subjected to real-time quantitative PCR analysis using TaqMan Gene Expression Assays and an Applied Biosystems 7500 real-time PCR system (Life Technologies) to quantitatively analyze mRNA expression levels. Relative expression levels for the specific transcripts were detected by normalizing with those from the *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) gene. To distinguish mRNA from the endogenous *Geminin* gene and that from exogenously transfected *Geminin* cDNA lacking a majority of the 3′-UTR except for 38 base pairs from the termination codon, the probe and the primer set for 3′-UTR of *Geminin* mRNA was determined by the Custom TaqMan Assay Design Tool (www5.appliedbiosystems.com/tools/cadt; Life Technologies).

Cell cycle analysis

Cell cycle analysis was performed with the APC BrdU-Flow Kit (BD PharMingen, San Diego, CA). Cell sorting analysis was performed on the FACSCalibur flow cytometer and FACSAria II cell sorter (BD Biosciences Immunocytometry Systems, San Jose, CA).

Cell culture and DNA transfection experiments

A mouse fibroblast cell line, NIH 3T3, and a human kidney cell line, HEK-293, were grown in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA). cDNAs or Flag-, HA-, and 6Myc-cDNAs were subcloned downstream of the cytomegalovirus promoter of pcDNA3.1 expression vector (Life Technologies; Ohno *et al.*, 2010). Geminin5EQ (Seo *et al.*, 2005) was generated by PCR-mediated mutagenesis with the mouse cDNA for *Geminin* by using the primer pair 5′-AGAATTAAATTGCTGACTATCCGGTGATTC-3′ and 5′-CAACAACAAGCTGTTGAGTATTCAGAACTG-3′ and PfuTurbo Hotstart DNA polymerase (Agilent Technologies, Santa Clara, CA). The PCR product was tail-phosphorylated and tail-to-tail ligated by using T4 polynucleotide kinase and T4 DNA ligase (Takara Bio, Otsu, Japan). Plasmid DNAs were transfected into NIH 3T3 cells by using Lipofectamine 2000 (Life Technologies) and into HEK 293 cells by the calcium phosphate coprecipitation method (Ohno *et al.*, 2010). Twelve hours after transfection, the cells were washed and then serum deprived for 24 h. The resultant transfectants were subjected to further analyses. The total amount of plasmid DNA for transfection was adjusted to the constant amount by adding an empty vector. Next, the cells were lysed and subjected to a luciferase assay with the Dual-Glo luciferase assay system (Promega, Madison, WI) according to the manufacturer's protocol. We used the pGL3-basic firefly luciferase reporter vector incorporated with the promoter regions of the human *Geminin* gene (Yoshida and Inoue, 2004) and a *Renilla* luciferase reporter plasmid, pE2MTx4-*Renilla* (Ohtani *et al.*, 2000), as internal control to standardize transfection efficiency. pE2MTx4-*Renilla* carries the pGL2 promoter, four tandem repeats of the adenovirus E2 enhancer, and two copies

of mutated E2F-binding sites. All assays were performed at least three times.

siRNA experiments

NIH 3T3 cells were transfected with the following double-strand (ds) RNAs (Thermo Fisher Scientific) at 40 nM by using Lipofectamine RNAiMAX (Life Technologies): Geminin, a mixture of the four dsRNAs GGAGUCAUUUGAUCUUAUG, GAGACUGAAUGGUGAACCU, AGAAGUAGCAGAACAUGUA, and UUGAAUCACUGGAUAAUCA; and E2F1, a mixture of the four dsRNAs GCCAAGAAGUCCAAGAAUC, GGAGAGUGCAGACGGGAUU, GCUAUGAAACCUCACUAAA, and CCACGAGGCCCUUGACUAU. siPerfect Negative Control (Sigma-Aldrich, St. Louis, MO) was transfected at the same concentration as a nontarget negative control. siRNA transfection was done for 24 h, and the cells were subjected to further analyses (Ohno *et al.*, 2010).

Immunoprecipitation and immunoblot analysis

Cell extracts were obtained by resuspending cell pellets in RIPA buffer consisting of 10% glycerol, 0.5% Triton X-100, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1.5 mM $MgCl_2$, and a protease inhibitor cocktail, Complete Mini (Roche Diagnostics, Mannheim Germany), sonicated for 30 s on ice, and centrifuged for 15 min at 15,000 × *g*. The supernatant of the lysate was subjected to immunoprecipitation experiments and the lysate to immunoprecipitation with GammaBind G Sepharose (GE Healthcare, Milwaukee, WI). Proteins were separated by SDS–PAGE, transferred to Immobilon-P (Millipore, Billerica, MA), immunoblotted with primary antibodies, and visualized with horseradish peroxidase–conjugated anti-rabbit IgG and SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific; Ohno *et al.*, 2010, 2013).

ChIP assay

ChIP assay was performed by using a LowCell#ChIP Kit (Diagenode, Liege, Belgium) according to the manufacturer's instructions (Solomon *et al.*, 1988; Yasunaga *et al.*, 2013). Freshly prepared NIH 3T3 cells (~1×10^6) were fixed with 0.96% formaldehyde for 8 min at room temperature; this was terminated by addition of 1.25 M glycine. DNA–protein cross-linked cells were washed twice with cold phosphate-buffered saline and treated with lysis buffer supplemented with 20 mM sodium butylate for 5 min on ice. The samples were then subjected to sonication to shear the chromatin using the Handy Sonic UR-20P (Tomy Digital Biology, Tokyo, Japan) for 18 cycles (20 s ON, 40 s OFF). The average size of DNA fragments was confirmed to be ~500 base pairs, ranging from 200 to 1000 base pairs. The sheared chromatin was incubated with protein A– or G–coated paramagnetic beads bound with an antibody of interest listed in Supplemental Table 1 (anti-E2F1, anti-HA, anti-H3ac, anti-H3K4me3, anti-H3K27me27, anti-H2AK119ub, anti-Scmh1, anti-Ring1B, anti-Bmi1, and anti-Rae28 antibodies) overnight at 4°C. The samples were then washed and immunoprecipitated. The immunoprecipitate was incubated at 55°C for 15 min and boiled for 15 min, and DNA was then purified by using supplied DNA isolation buffer. ChIP DNA was examined by standard PCR for detecting genomic control A region (Cont-A) and E2F-R in the *Geminin* gene. PCR primer pairs used were as follows: Cont-A, 5′-CTTCCCGACTCTGAGGACTG-3′ and 5′-AGAACTAGGGCCAAGGGAAC-3′; and E2F-R, 5′-GAGTCTGGGGACTTGAAAGG-3′ and 5′-GGGAGGGATCTACACCCAGT-3′. PCR conditions were (95°C, 5 min) × 1 cycle for predenaturating and (95°C, 10 s; 63°C, 30 s; 72°C, 1 min) × 35 cycles and (72°C, 1 min) × 1 cycle for extension.

Nuclease accessibility analysis

Accessibility to nuclease digestion was examined by using EpiQ chromatin analysis kit (Bio-Rad, Hercules, CA) following the manufacturer's protocol with minor modifications (Yellajoshyula *et al.*, 2011; Ito *et al.*, 2013). Briefly, 1×10^6 NIH 3T3 cells were permeabilized with EpiQ chromatin buffer. The cells were subjected to in situ chromatin digestion with EpiQ nuclease for 1 h at 37°C. The digestion was terminated with EpiQ stop buffer. Genomic DNA was extracted by using DNA lysis solution and washed with low/high-stringency wash solution in the column. Extracted DNA was eluted from the column with DNA elution solution and subjected to PCR analysis for E2F-R with the primer sets described. Nuclease accessibility is defined as the susceptibility of the targeted genomic DNA region to nuclease digestion. The index of the target genomic DNA region after nuclease treatment is calculated relative to that of the reference gene region of the *Rhodopsin* (*Rho*) gene that is not susceptible to nuclease digestion. The data were analyzed by using EpiQ Chromatin Kit Data Analysis Tool (www.bio-rad.com/epiq; Bio-Rad).

Statistical analysis

More than three independent experiments were done, and the data were analyzed using the Student's *t* test. The results are shown with SEM.

Antibodies

Primary and secondary antibodies used are listed in Supplemental Table 1.

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REFERENCES

Ballabeni A, Melixetian M, Zamponi R, Masiero L, Marinoni F, Helin K (2004). Human geminin promotes pre-RC formation and DNA replication by stabilizing CDT1 in mitosis. EMBO J 23, 3122–3132.

Benjamin JM, Torke SJ, Demeler B, McGarry TJ (2004). Geminin has dimerization, Cdt1-binding, and destruction domains that are required for biological activity. J Biol Chem 279, 45957–45968.

Blow JJ, Hodgson B (2002). Replication licensing—defining the proliferative state? Trends Cell Biol 12, 72–78.

Dahiya A, Wong S, Gonzalo S, Gavin M, Dean DC (2001). Linking the Rb and Polycomb pathways. Mol Cell 8, 557–659.

Danielian PS, Friesenhahn LB, Faust AM, West JC, Caron AM, Bronson RT, Lees JA (2008). E2f3a and E2f3b make overlapping but different contributions to total E2f3 activity. Oncogene 27, 6561–6570.

DeGregori J, Leone G, Miron A, Jakoi L, Nevins JR (1997). Distinct roles for E2F proteins in cell growth control and apoptosis. Proc Natl Acad Sci USA 94, 7245–7250.

Del Bene F, Tessmar-Raible K, Wittbrodt J (2004). Direct interaction of geminin and Six3 in eye development. Nature 427, 745–749.

Dunaief JL, Strober BE, Guha S, Khavari PA, Alin K, Luban J, Begemann M, Crabtree GR, Goff SP (1994). The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. Cell 79, 119–130.

Gonzalez MA, Tachibana KE, Adams DJ, van der Weyden L, Hemberger M, Coleman N, Bradley A, Laskey RA (2006). Geminin is essential to prevent endoreduplication and to form pluripotent cells during mammalian development. Genes Dev 20, 1880–1884.

Hara K, Nakayama KI, Nakayama K (2006). Geminin is essential for the development of preimplantation mouse embryos. Genes Cells 11, 1281–1293.
Ito Y, Nativio R, Murrell A (2013). Induced DNA demethylation can reshape chromatin topology at the IGF2-H19 locus. Nucleic Acids Res 41, 5290–5302.
Karamitros D, Kotantaki P, Lygerou Z, Veiga-Fernandes H, Pachnis V, Kioussis D, Taraviras S (2010). Differential geminin requirement for proliferation of thymocytes and mature T cells. J Immunol 184, 2432–2441.
Kroll KL, Salic AN, Evans LM, Kirschner MW (1998). Geminin, a neuralizing molecule that demarcates the future neural plate at the onset of gastrulation. Development 125, 3247–3258.
Lee C, Hong B, Choi JM, Kim Y, Watanabe S, Ishimi Y, Enomoto T, Tada S, Kim Y, Cho Y (2004). Structural basis for inhibition of the replication licensing factor Cdt1 by geminin. Nature 430, 913–917.
Leone G, Nuckolls F, Ishida S, Adams M, Sears R, Jakoi L, Miron A, Nevins JR (2000). Identification of a novel E2F3 product suggests a mechanism for determining specificity of repression by Rb proteins. Mol Cell Biol 20, 3626–3632.
Luo L, Yang X, Takihara Y, Knoetgen H, Kessel M (2004). The cell-cycle regulator geminin inhibits Hox function through direct and Polycomb-mediated interactions. Nature 427, 749–753.
Lutzmann M, Maiorano D, Méchali M (2006). A Cdt1-geminin complex licenses chromatin for DNA replication and prevents rereplication during S phase in *Xenopus*. EMBO J 25, 5764–5774.
Markey M, Siddiqui H, Knudsen ES (2004). Geminin is targeted for repression by the retinoblastoma tumor suppressor pathway through intragenic E2F sites. J Biol Chem 279, 29255–29262.
McGarry TJ, Kirschner MW (1998). Geminin, an inhibitor of DNA replication, is degraded during mitosis. Cell 93, 1043–1053.
Muchardt C, Yaniv M (2001). When the SWI/SNF complex remodels…the cell cycle. Oncogene 20, 3067–3075.
Nagl NG Jr, Wang X, Patsialou A, Van Scoy M, Moran E (2007). Distinct mammalian SWI/SNF chromatin remodeling complexes with opposing roles in cell-cycle control. EMBO J 26, 752–763.
Narlikar GJ, Fan HY, Kingston RE (2002). Cooperation between complexes that regulate chromatin structure and transcription. Cell 108, 475–487.
Ohno Y, Yasunaga S, Janmohamed S, Ohtsubo M, Saeki K, Kurogi T, Mihara K, Iscove NN, Takihara Y (2013). Hoxa9 transduction induces hematopoietic stem and progenitor cell activity through direct down-regulation of Geminin protein. PLoS One 8, e53161.
Ohno Y, Yasunaga S, Ohtsubo M, Mori S, Tsumura M, Okada S, Ohta T, Ohtani K, Kobayashi M, Takihara Y (2010). Hoxb4 transduction down-regulates Geminin protein, providing hematopoietic stem and progenitor cells with proliferation potential. Proc Natl Acad Sci USA 107, 21529–21534.
Ohtani K, Iwanaga R, Arai M, Huang Y, Matsumura Y, Nakamura M (2000). Cell type-specific E2F activation and cell cycle progression induced by the oncogene product Tax of human T-cell leukemia virus type I. J Biol Chem 275, 11154–11163.
Ohtsubo M *et al.* (2008). Polycomb-group complex 1 acts as an E3 ubiquitin ligase for Geminin to sustain hematopoietic stem cell activity. Proc Natl Acad Sci USA 105, 10396–10401.
Quinn LM, Herr A, McGarry TJ, Richardson H (2001). The *Drosophila* Geminin homolog: roles for Geminin in limiting DNA replication, in anaphase and in neurogenesis. Genes Dev 15, 2741–2754.
Saxena S, Dutta A (2005). Geminin-Cdt1 balance is critical for genetic stability. Mutat Res 569, 111–121.
Saxena S, Yuan P, Dhar SK, Senga T, Takeda D, Robinson H, Kornbluth S, Swaminathan K, Dutta A (2004). A dimerized coiled-coil domain and an adjoining part of geminin interact with two sites on Cdt1 for replication inhibition. Mol Cell 15, 245–258.
Schulze A, Zerfass K, Spitkovsky D, Middendorp S, Bergès J, Helin K, Jansen-Dürr P, Henglein B (1995). Cell cycle regulation of the cyclin A gene promoter is mediated by a variant E2F site. Proc Natl Acad Sci USA 92, 11264–11268.
Seo S, Herr A, Lim JW, Richardson GA, Richardson H, Kroll KL (2005). Geminin regulates neuronal differentiation by antagonizing Brg1 activity. Genes Dev 19, 1723–1734.
Shinnick KM, Eklund EA, McGarry TJ (2010). Geminin deletion from hematopoietic cells causes anemia and thrombocytosis in mice. J Clin Invest 120, 4303–4315.
Solomon MJ, Larsen PL, Varshavsky A (1988). Mapping protein-DNA interactions in vivo with formaldehyde: evidence that histone H4 is retained on a highly transcribed gene. Cell 53, 937–947.
Stevens C, La Thangue NB (2003). E2F and cell cycle control: a double-edged sword. Arch Biochem Biophys 412, 157–169.
Suganuma T, Workman JL (2008). Crosstalk among histone modifications. Cell 135, 604–607.
Tada S, Li A, Maiorano D, Méchali M, Blow JJ (2001). Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin. Nat Cell Biol 3, 107–113.
Takahashi Y, Rayman JB, Dynlacht BD (2000). Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. Genes Dev 14, 804–816.
Takihara Y (2008). Role of Polycomb-group genes in normal and malignant stem cells. Int J Hematol 87, 25–34.
Takihara Y (2011). Role for Geminin in sustaining the activity of hematopoietic stem cells. Cell Cycle 10, 561–562.
Tsunematsu T, Takihara Y, Ishimaru N, Pagano M, Takata T, Kudo Y (2013). Aurora-A controls pre-replicative complex assembly and DNA replication by stabilizing geminin in mitosis. Nat Commun 4, 1885.
Tyagi S, Chabes AL, Wysocka J, Herr W (2007). E2F activation of S phase promoters via association with HCF-1 and the MLL family of histone H3K4 methyltransferases. Mol Cell 27, 107–119.
van den Heuvel S, Dyson NJ (2008). Conserved functions of the pRB and E2F families. Nat Rev Mol Cell Biol 9, 713–724.
Wang L, Brown JL, Cao R, Zhang Y, Kassis JA, Jones RS (2004). Hierarchical recruitment of polycomb group silencing complexes. Mol Cell 14, 637–646.
Wohlschlegel JA, Dwyer BT, Dhar SK, Cvetic C, Walter JC, Dutta A (2000). Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. Science 290, 2309–2312.
Wolffe A (1998). Chromatin and nuclear assembly. In: Chromatin: Structure and Function, 3rd ed., San Diego, CA: Academic Press, 173–239.
Yang VS, Carter SA, Hyland SJ, Tachibana-Konwalski K, Laskey RA, Gonzalez MA (2011). Geminin escapes degradation in G1 of mouse pluripotent cells and mediates the expression of Oct4, Sox2, and Nanog. Curr Biol 21, 692–699.
Yasunaga S *et al.* (2013). Scmh1 has E3 ubiquitin ligase activity for Geminin and histone H2A and regulates Geminin stability directly or indirectly via transcriptional repression of Hoxa9 and Hoxb4. Mol Cell Biol 33, 644–660.
Yellajoshyula D, Lim JW, Thompson DM Jr, Witt JS, Patterson ES, Kroll KL (2012). Geminin regulates the transcriptional and epigenetic status of neuronal fate-promoting genes during mammalian neurogenesis. Mol Cell Biol 32, 4549–4560.
Yellajoshyula D, Patterson ES, Elitt MS, Kroll KL (2011). Geminin promotes neural fate acquisition of embryonic stem cells by maintaining chromatin in an accessible and hyperacetylated state. Proc Natl Acad Sci USA 108, 3294–3299.
Yoshida K, Inoue I (2004). Regulation of Geminin and Cdt1 expression by E2F transcription factors. Oncogene 23, 3802–3812.
Zhang HS, Gavin M, Dahiya A, Postigo AA, Ma D, Luo RX, Harbour JW, Dean DC (2000). Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. Cell 101, 79–89.