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 nucleosome 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, GemininSEQ, which does not interact with Brahma or Brg1, did not suppress accessibility to nucleosome 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.

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...
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 (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 Geminin5E (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 Geminin5E 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 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 nucleosome 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.](image-url)
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...
Geminin specifically regulates E2F1-mediated transcriptional activation in NIH 3T3 cells (Figure 5B). Here knockdown of E2F1 and supertransfection of 6Myc-Geminin, Flag-GemininSEQ, 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 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-GemininSEQ, 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-GemininSEQ were detected by anti-HA and anti-Geminin antibodies, respectively. Statistical significance is as follows: **P < 0.05.

**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.
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 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 chromatin 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).

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.

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.
abrogated the molecular interaction of 6Myc-Geminin with Flag-Brahma-DomII or Flag-Brg1-DomII in a dose-dependent manner, suggesting that Geminin5EQ overexpression preempts the molecular interaction of wild-type Geminin with Brahma/Brig1 in a dominant-negative manner (Figure 8A 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/Brig1, and Geminin5EQ showed the augmented reporter activity synergistically with E2F3a-7 (Figure 2B). We confirmed that overexpression of HA-E2F5 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 transfecants with HA-E2F1 and HA-Geminin5EQ (Figure 6B). Finally, a moderate increase in H3ac and H3K4me3 was detected in transfecants with HA-Geminin5EQ only (Figure 6B), and nuclease accessibility in E2F-R was augmented in the Flag-Geminin5EQ transfecants (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, Rael28, and Scml1 (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
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 in 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 through direct inhibition of the chromatin remodeling factors 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 transcription 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 (Yellajosyula 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 G0/G1 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 (ZYM0 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 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). GemininSEQ (Seo et al., 2005) was generated by PCR-mediated mutagenesis with the mouse cDNA for Geminin by using the primer pair 5′-AGAATTTAATTGCAGACCTCCTGGT-GATTCC-3′ and 5′-CAAAAGAACCTGTTAGTATCTAGAAGGTTCTG3′ 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 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 GGAGUGCCAGUAAU; GGACUGAAA-UGGGUACCUCAG, AGAGAUGACAGAAACUGUA, and UUGGAAU-CACUGGAAUAUCA; and E2F1, a mixture of the four dsRNAs GCCAAAGAGGCUCAAAGACAUC, GGAGUGUAGAGCGGAAU, GCUAUGAAACUCACUAAA, and CACCGAGGCCCUUUGACUAA. 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 MgCl₂, 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 horse-radish 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 × 10⁹) 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 estimated 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-Scm1h1, anti-Ring1B, anti-Bmi1, and anti-Rae28 antibodies) overnight at 4°C. The supernatant of the lysate was 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 estimated 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-Scm1h1, 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 DNA region targeted by antibodies. Nuclease accessibility analysis

Accessibility to nuclease digestion was examined by using EpiQ Chomatin 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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