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## BRIEF REPORT

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# Identification and characterization of an enhancer element regulating expression of *Cdkn1c* (p57 gene)

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## Abstract

The mammalian p57 protein is a member of the CIP/KIP family of cyclin-dependent kinase inhibitors and plays an essential role in the development of multiple tissues during embryogenesis as well as in the maintenance of tissue stem cells in adults. Although several transcription factors have been implicated in regulating the p57 gene, cis-elements such as enhancers that regulate its expression have remained ill-defined. Here we identify a candidate enhancer for the mouse p57 gene (*Cdkn1c*) within an intron of the *Kcnq1* locus by 4C-seq analysis in mouse embryonic stem cells (mESCs). Deletion of this putative enhancer region with the CRISPR-Cas9 system or its suppression by CRISPR interference resulted in a marked attenuation of *Cdkn1c* expression in differentiating mESCs. Our results thus suggest that this region may serve as an enhancer for the p57 gene during early mouse embryogenesis.

## KEYWORDS

embryonic stem cell, enhancer, gene expression, p57

## 1 | INTRODUCTION

The cell cycle is a fundamental biological process of all organisms, with its progression being driven by sequential activation of phase-specific cyclin–cyclin-dependent kinase (CDK) complexes (Nurse, 2000). The activity of cyclin-CDK complexes is suppressed by CDK inhibitors (CKIs) through direct protein–protein interaction (Sherr & Roberts, 1999). Proper control of the timing and rate of the cell cycle by CKIs is integral to maintenance of tissue homeostasis as well as to developmental processes in mammals (Nakayama & Nakayama, 1999; Pfeuty, 2012; Reis & Edgar, 2004), and its dysregulation can give rise to various congenital abnormalities (Nakayama et al., 1996;

Takahashi et al., 2000) or to cancer (Bury et al., 2021; Malumbres & Barbacid, 2009). Mammalian CKIs are divided into two primary families: Members of the inhibitors of CDK4 (INK4) family specifically target CDK4- or CDK6–cyclin D complexes, whereas members of the CDK-interacting protein/kinase-inhibitory protein (CIP/KIP) family inhibit a broader spectrum of cyclin-CDK complexes (Sherr & Roberts, 1999).

Among the three members of the CIP/KIP family of CKIs—p21, p27, and p57—p57 plays a key role in early developmental processes in mammals. The p57 protein is encoded by a paternally imprinted gene, and its downregulation as a result of genetic mutations or imprinting dysregulation in humans gives rise to Beckwith–Wiedemann

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syndrome (BWS) (Bhuiyan et al., 1999; Hatada et al., 1996; Weksberg et al., 2010), a syndrome of overgrowth characterized by macrosomia, visceromegaly, and abdominal defects. The p57 protein is expressed at higher levels during embryogenesis than in adult tissues in mice (Creff & Besson, 2020; Nagahama et al., 2001), and its expression pattern is mutually exclusive relative to that of p27 in several embryonic tissues (Nagahama et al., 2001; Nakayama & Nakayama, 1999). Whereas mice lacking p21 or p27 do not manifest any lethal phenotypes (Deng, 1995; Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996), p57-deficient mice develop cleft palate and bone malformations, with most undergoing neonatal death as a result of severe dyspnea (Takahashi et al., 2000; Yan et al., 1997; Zhang et al., 1997). In addition to its importance for early development, p57 is specifically expressed in adult tissue stem cells including neural (Furutachi et al., 2013), hematopoietic (Matsumoto et al., 2011), intestinal (Higa et al., 2022), and cancer (Oka et al., 2023) stem cells, in which it plays a pivotal role in the maintenance of quiescence and stemness.

Although these findings suggest that precise control of p57 expression is required for both early and postnatal development, the detailed molecular mechanisms underlying such regulation have remained unclear. In particular, although transcription factors that control expression of the p57 gene in various cell types have been identified (Rossi, 2018), as have enhancer regions for the gene in the placenta (López-Abad et al., 2016), glucocorticoid-treated cancer cell lines (Prekovic et al., 2021), and skin fibroblasts derived from individuals with BWS (Naveh et al., 2021), knowledge of the cis-regulatory elements of the gene has remained limited.

We have now identified a putative enhancer of the p57 gene (*Cdkn1c*) shared by mouse embryonic stem cells (mESCs) in the naïve state and those induced to differentiate with all-*trans* retinoic acid (ATRA). Deletion or functional suppression of this enhancer resulted in marked attenuation of the increase in p57 gene expression associated with ATRA-induced differentiation. Our results thus suggest that a constitutive enhancer may function during the substantial elevation of p57 expression apparent during early mouse embryogenesis.

## 2 | RESULTS

### 2.1 | The abundance of p57 mRNA in mESCs is increased by ATRA treatment

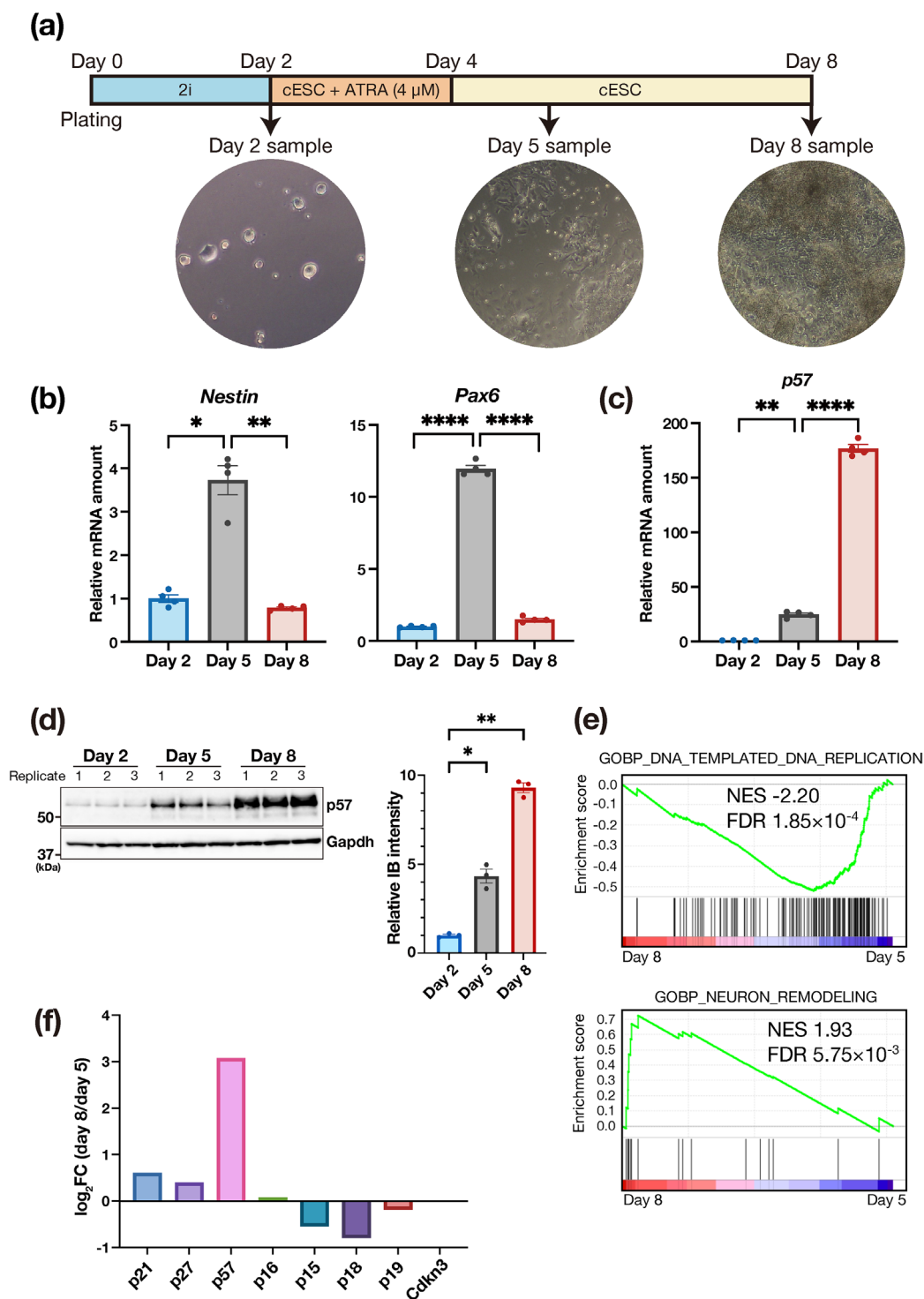
To identify enhancers that might regulate p57 gene expression during early embryogenesis, we subjected

differentiating mESCs to circular chromatin conformation capture-sequencing (4C-seq) analysis (Krijger et al., 2020). Given that ATRA was previously shown to induce the neural differentiation of mESCs (Kubo et al., 2021), we first investigated the effects of ATRA treatment on the differentiation state of and p57 expression in these cells. We cultured mESCs in 2i medium (Ying et al., 2008) for 2 days and then exposed the cells to 4  $\mu$ M ATRA in medium containing fetal bovine serum (FBS) for an additional 2 days. The cells were then further cultured in medium without ATRA (Figure 1a). Reverse transcription (RT) and quantitative polymerase chain reaction (qPCR) analysis after 5 days of culture revealed a significant increase in the expression of Nestin and Pax6 genes (Figure 1b), both of which are representative marker genes for early neural lineage cells, indicating that ATRA indeed triggered the neural differentiation of mESCs. More prolonged culture showed that the cells achieved confluence by day 8 (Figure 1a), resulting in an almost complete cessation of their proliferation. At this time point, however, the expression of Nestin and Pax6 genes had returned to levels similar to those apparent in the 2i-cultured naïve mESCs.

The abundance of p57 mRNA and protein in mESCs was markedly increased in association with ATRA-induced differentiation, being highest at day 8 (Figure 1c, d). RNA-seq analysis of the cells after culture for 2, 5, or 8 days revealed substantial differences in gene expression profiles among the three time points. Gene set enrichment analysis (GSEA) for comparison of cells at day 8 with those at day 5 showed that cell cycle-related genes were suppressed and marker genes for neuron remodeling during neuron maturation in nervous system development were upregulated at day 8 (Figure 1e). Furthermore, the fold change in p57 mRNA abundance at day 8 compared with day 5 was markedly greater than that for any other CKI mRNA (Figure 1f). These results suggested that p57 plays a primary role both in cell cycle regulation and in the progression of tissue development.

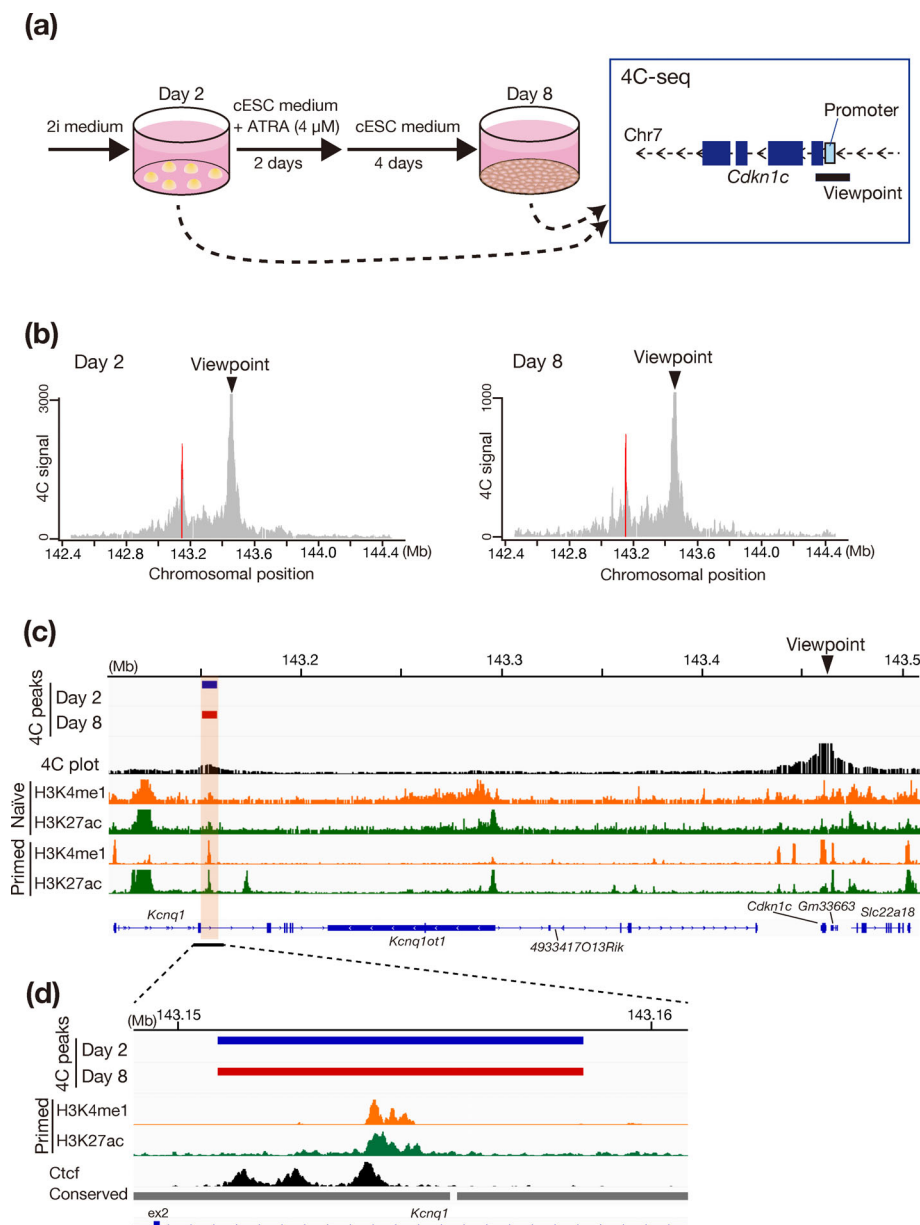
### 2.2 | Identification of a putative *Cdkn1c* enhancer region at the *Kcnq1* locus

To explore the molecular mechanism underlying the upregulation of p57 mRNA by ATRA treatment in mESCs, we searched for a putative enhancer element that might physically link to the *Cdkn1c* promoter in day 2 and day 8 cells by 4C-seq analysis (Figure 2a). Application of peakC software (Geeven et al., 2018) to the results of 4C-seq analysis revealed a single common peak located within intron 2 of the *Kcnq1* gene in both the day 2 and day 8 samples, with the intensity of this peak being



**FIGURE 1** Characterization of differentiating mESCs treated with ATRA. (a) Experimental protocol for analysis of mESC differentiation. The photos shown below the time course of cell culture are phase-contrast microscopic images of mESCs at days 2, 5, and 8. cESC, complete ESC medium. (b) and (c) RT-qPCR analysis of early neural lineage gene expression (b) and of p57 mRNA abundance (c) in mESCs at days 2, 5, and 8. Data were normalized by the expression of *Hprt* and are means  $\pm$  SEM from four independent experiments. \* $p < .05$ , \*\* $p < .01$ , \*\*\*\* $p < .0001$  (Welch's ANOVA test). (d) Immunoblot analysis of p57 protein abundance at days 2, 5, and 8. Left: Representative immunoblot chemiluminescence images of triplicate for each time point. Right: Quantification of p57 immunoblot intensity.  $n = 3$  for each time point (e) GSEA plots for the indicated gene sets and RNA-seq data from mESCs at day 8 versus those at day 5. NES, normalized enrichment score; FDR, false discovery rate. (f) Fold change (FC) in CKI gene expression for mESCs at day 8 relative to those at day 5 as determined from RNA-seq data.

**FIGURE 2** Identification of a putative enhancer region for *Cdkn1c* within intron 2 of *Kcnq1* by 4C-seq analysis. (a) Scheme for sample preparation for 4C-seq analysis. (b) 4C-seq peak plots for the day 2 and day 8 samples as generated with peakC software. The red vertical lines indicate statistically significant peaks. (c) Screenshot for IGV software displaying six traces. The top two traces show 4C peaks for the day 2 and day 8 samples. The third trace is a raw 4C signal plot for one replicate of day 2 cells. The fourth and fifth traces show ChIP-seq signals for active enhancer marks (H3K4me1 and H3K27ac) in the naïve state mESCs, cultured in 2i medium, acquired from ChIP-Atlas. The sixth and seventh traces show ChIP-seq signals for active enhancer marks in the primed state mESCs cultured in FCS-containing medium. The last trace indicates gene loci. (d) Enlarged section of the traces in (c). Two traces above the bottom gene loci indicate ChIP-seq signals for Ctf, and conserved regions (acquired from the UCSC genome browser) between the human and mouse genomes, respectively. ex2, exon 2.



almost identical at both time points (Figure 2b). Unfortunately, however, we were unable to find a specific peak that was present only in the day 8 cells. These results suggested that the identified region may constitutively interact with the *Cdkn1c* promoter in both naïve and differentiated mESCs, with the extent of the interaction changing little during the differentiation process.

We next investigated the functional role of this putative enhancer region in epigenetic regulation of *Cdkn1c* expression with the use of ChIP-Atlas, a public repository of chromatin immunoprecipitation (ChIP)-seq data for histone modifications (Oki et al., 2018). Application of ChIP-Atlas for the region surrounding the peak detected by 4C-seq analysis revealed that active enhancer marks including monomethylated lysine-4 (H3K4me1) and acetylated lysine-

27 (H3K27ac) of histone H3 were enriched in this region of interest in mESCs cultured in FCS-containing medium, which is referred to as the primed state (Figure 2c), consistent with the notion that this region might function as a constitutive enhancer that interacts with the *Cdkn1c* promoter and facilitates its transcription. In the mESCs cultured in 2i medium, referred to as the naïve state, we observed less significant enrichment of those active enhancer marks, which suggests that this region functions as a stronger enhancer in differentiating mESCs than in naïve state mESCs. This region was also found to be highly conserved in the human genome (Figure 2d) (Cerrato et al., 2014), suggestive of its functional importance. These results thus implicated the region detected by 4C-seq analysis as an enhancer for p57 gene expression.

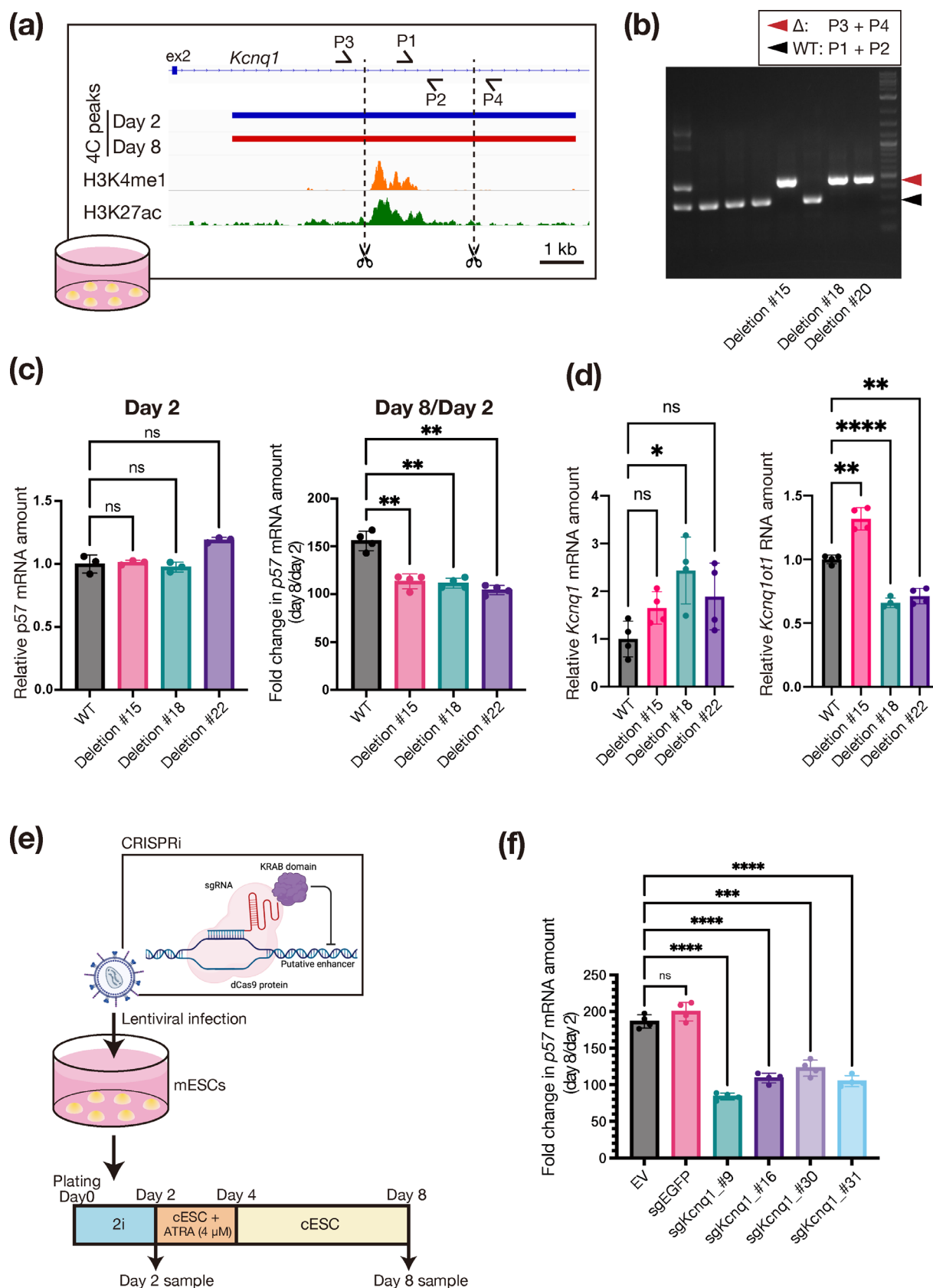


FIGURE 3 Legend on next page.

## 2.3 | Suppression of the putative enhancer attenuates upregulation of *Cdkn1c*

To examine the functional relevance of the putative *Cdkn1c* enhancer, we deleted this region in mESCs with the use of the CRISPR/Cas9 system (Figure 3a). Given that the identified active enhancer marks were located in the central region of the 4C-seq peak, we deleted this central portion in mESCs. The deletion was confirmed by genomic PCR analysis (Figure 3b) and genomic sequencing. We obtained three mESC clones homozygous for the induced deletion. RT-qPCR analysis revealed no difference in the abundance of p57 mRNA between the mutant clones and the parental wild-type (WT) cells under the 2i culture condition (Figure 3c). On the other hand, the ATRA-induced increase in p57 mRNA abundance was partially but significantly attenuated in all mutant clones (Figure 3c), indicating that the putative enhancer region is required for the differentiation-associated upregulation of p57 gene expression but is dispensable for maintenance of the basal expression of *Cdkn1c* in ground-state mESCs. The mRNA levels of *Kcnq1* differed in each clone, but the difference against WT control was not significant overall (Figure 3d). The levels of *Kcnq1ot1*, the non-coding RNA that suppresses the expression of p57 in the paternal allele, showed significant variance among the clones (Figure 3d), however, this was unlikely to affect p57 expression because the directions of difference were inconsistent.

To further validate that the identified region serves as a cis-acting epigenetic regulator, we applied the CRISPR interference (CRISPRi) system (Thakore et al., 2015) to inhibit its putative activity. We introduced four independent single guide RNAs (sgRNAs) targeting this region in combination with the dCas9-KRAB fusion protein into mESCs and then cultured the cells consecutively with ATRA for 2 days and in ATRA-free medium for 4 days

(Figure 3e). CRISPRi of this region resulted in a significant reduction in the extent of the ATRA-induced increase in p57 mRNA abundance compared with that apparent in control cells (Figure 3f), consistent with the effect of genetic deletion of this region (Figure 3c). Collectively, our data thus suggest that the putative enhancer region located downstream of exon 2 of *Kcnq1* indeed functions as an epigenetic cis-activator of *Cdkn1c* transcription during early mouse development.

## 3 | DISCUSSION

We have here identified an enhancer region that may regulate *Cdkn1c* (p57 gene) expression in the early stage of mouse embryogenesis. Genetic ablation or CRISPRi-mediated functional suppression of this region resulted in a significant downregulation of p57 gene expression in differentiating mESCs, without an effect on basal expression of the gene in naïve mESCs.

An enhancer for p57 gene expression has previously been proposed to be located within the *KCNQ1* gene locus in both the human and mouse genomes (Cerrato et al., 2014; John et al., 2001). Although conservation of the genomic sequence in this region among species and enrichment of active enhancer marks as revealed by ChIP-seq analysis have supported this proposal (Cerrato et al., 2014), direct evidence has been lacking.

Direct interaction of the p57 gene promoter with a region in the *KCNQ1* locus has been demonstrated in human placental cells (López-Abad et al., 2016). In human skin fibroblasts, a region within intron 2 of *KCNQ1* interacts simultaneously with the p57 gene promoter and the 5' end of the *KCNQ1* gene, whereas these interactions are attenuated in cells from individuals with BWS (Naveh et al., 2021). This region appears to be identical to the region identified in the present study, despite the difference in species. However, the actual function of

**FIGURE 3** Suppression of the putative enhancer region attenuates *Cdkn1c* expression in differentiating mESCs. (a) Scheme for CRISPR/Cas9-mediated deletion of the putative enhancer region in mESCs. The two vertical dashed lines indicate the 5' and 3' ends of the deleted sequence. Arrows show the positions of PCR primers for genotyping. (b) Agarose gel electrophoresis for genotyping PCR analysis of mutant mESCs generated as in (a). Arrowheads indicate the expected sizes of WT and mutant ( $\Delta$ ) DNA fragments. (c) RT-qPCR analysis of *Cdkn1c* expression in three mutant mESC lines (clones #15, #18, and #22) and the parental WT cells. The left panel shows the abundance of p57 mRNA in cells at day 2 (naïve state) of the culture protocol in Figure 1a, and the right panel shows the (ATRA-induced) increase in the amount of p57 mRNA at day 8 relative to day 2. (d) RT-qPCR analysis of *Kcnq1* and *Kcnq1ot1* expression in three mutant mESC lines (clones #15, #18, and #22) and the parental WT cells. The results are of the differentiated (day 8) mESCs. (e) Scheme for CRISPRi experiments. Both sgRNAs and the dCas9-KRAB fusion protein were introduced into naïve mESCs by lentiviral infection to suppress the activity of the putative enhancer region. (f) RT-qPCR analysis of the fold change in p57 mRNA abundance in CRISPRi-modified mESCs at day 8 relative to day 2 of the culture protocol in (d). As controls, mESCs subjected to CRISPRi without an sgRNA (EV) or with an sgRNA targeting enhanced green fluorescent protein (sgEGFP) were examined. sgKcnq1\_#9, \_#16, \_#30, and \_#31 indicate cells subjected to CRISPRi with *Kcnq1* sgRNAs directed to the corresponding positions of the target region. Data in (c) and (e) are means  $\pm$  SEM for four independent experiments. \*\* $p < .01$ , \*\*\* $p < .001$ , \*\*\*\* $p < .0001$ ; ns, not significant (Welch's ANOVA test).

this previously identified region as an enhancer was not assessed. We have now shown that ablation or functional suppression of the corresponding region in intron 2 of *Kcnq1* resulted in a significant downregulation of *Cdkn1c* expression during mESC differentiation. The fact that such an effect was not evident in the same mESC clones in the naïve state, in which the level of *Cdkn1c* expression is much lower than that in the differentiated state, supports the notion that this region regulates p57 gene expression during its activation.

An enhancer for p57 gene expression has also been localized to intron 15 of *KCNQ1* and shown to be activated in response to glucocorticoid treatment in human lung cancer cell lines (Prekovic et al., 2021). Expression of the p57 gene was attenuated by deletion of this region in intron 15, whereas deletion of two additional putative enhancers in intron 15 and downstream of the *KCNQ1* locus identified in this previous study did not affect p57 gene expression. These findings suggest that genomic interactions do not necessarily give rise to active regulation of gene expression, consistent with our results that deletion of the identified enhancer in naïve mESCs did not influence the level of *Cdkn1c* expression and emphasizing the importance of experimental intervention. Moreover, no region in intron 2 of *KCNQ1* was mentioned as a putative enhancer for the p57 gene in this previous study of lung cancer cell lines, highlighting the tissue- and state-specific function of enhancers and the consequent importance that tissue or cell type be taken into consideration for analysis of such function.

As far as we are aware, our study is the first to investigate an enhancer for p57 gene expression in the context of development. The identification of a regulatory element at the *Kcnq1* locus that positively controls *Cdkn1c* expression in differentiating mESCs provides insight into the complex regulatory system of p57 function. We found that, despite the presence of this intergenic interaction in both naïve and differentiating mESCs, deletion of the enhancer region attenuated *Cdkn1c* expression only in the latter. This finding suggests that the genomic structure that promotes *Cdkn1c* expression may be established early during mouse embryogenesis, such as at the two-cell stage, and that regulation of *Cdkn1c* expression is driven by other factors, such as the upregulation of transcription factors, in response to state-specific signals.

Our study has several limitations. The CRISPR-mediated suppression of the enhancer region in mESCs unavoidably targeted both alleles, which makes it difficult to exclude the possibility that deletion on the paternal allele affected the results, even though *Cdkn1c* is silenced on the paternal allele. In addition, our study was conducted only under in vitro conditions. It should be feasible in the future to examine the allele-specific effects

of this enhancer region on *Cdkn1c* expression by generating mice with a targeted deletion in the allele inherited from each parent.

In summary, our findings shed light on the regulation of p57 gene expression during early embryogenesis. Further characterization of such regulation should provide insight into the pathogenesis of disorders such as BWS and may inform the development of improved treatment options for such conditions.

## 4 | EXPERIMENTAL PROCEDURES

### 4.1 | Cell culture

Embryonic day-4 JM8 mESCs were maintained on plates coated with 0.1% gelatin (Sigma, Cat# G1890) and in 2i medium, a mixture of DMEM/F-12 (Gibco, Cat# 1132033) and Neurobasal Medium (Gibco, Cat# 21103049) supplemented with B27 (Thermo Fisher Scientific, Cat# 17504), N-2 (Thermo Fisher Scientific, Cat# 17502), 3  $\mu$ M CHIR-99021 (Axon, Cat# 1386), 1  $\mu$ M PD033259010 (Cayman, Cat# 13034), leukemia inhibitory factor at 1000 U/mL (Sigma Aldrich, Cat# ESG1107), 0.05% bovine serum albumin (Gibco, Cat# 15260037), 2 mM L-glutamine (Gibco, Cat# 25030081), and 50 U/mL penicillin–streptomycin (Gibco, Cat# 15070063). For induction of cell differentiation, the culture medium was replaced with cESC medium, which consisted of DMEM (Gibco, Cat# 11965118) supplemented with 15% FBS (Gibco, Cat# 12483), nonessential amino acids (Gibco, Cat# 11140050), 1 mM sodium pyruvate (Gibco, Cat# 11360070), 2 mM L-glutamine, and 50 U/mL penicillin–streptomycin and was supplemented with 4  $\mu$ M ATRA as indicated. The cells were cultured in an incubator at 37°C with 20% O<sub>2</sub> and 5% CO<sub>2</sub>.

### 4.2 | RT-qPCR analysis

Cultured cells were washed with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free phosphate-buffered saline, after which 1 mL of Isogen (Nippon Gene, Cat# 311-02501) was added directly to the cells in each well of a 6- or 24-well plate for extraction and purification of RNA. RT was performed with the use of ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Cat# FSQ-301). The resulting cDNA was subjected to real-time PCR analysis with Luna Universal qPCR Master Mix (New England BioLabs, Cat# M3003) and specific primers in Step One Plus Real-Time PCR System (Applied Biosystems). The abundance of target mRNAs was normalized by that of hypoxanthine phosphoribosyl transferase (Hprt) mRNA. The sequences

of the primers (sense and antisense, respectively) were 5'-GGTTAAGCAGTACAGCCCCA-3' and 5'-TCCAA-CACTTCGAGAGGTCC-3' for Hprt, 5'-GCGCAAACG TCTGAGATGAGT-3' and 5'-AGAGTTCTTCCATCGTCC GCT-3' for p57, 5'-CTACCAGGAGCGGTGGC-3' and 5'-TCCACAGCCAGCTGGAATT-3' for Nestin, 5'-TAC CAGTGTCTACCAGCCAAT-3' and 5'-TGCACGAGTAT GAGGAGGTCT-3' for Pax6, 5'-TCACCGTCTTCCTCAT TGTTCT-3' and 5'-CCAGAGGCGGACCACATATT-3' for Kcnq1, and 5'-GCACTCTGGGTCTGTCTC-3' and 5'-CACTTCCCTGCCTCCTACAC-3' for Kcnq1ot1.

### 4.3 | Immunoblot analysis

Cells were lysed in ice-cold lysis buffer [10 mM Tris-HCl (pH 7.5)], 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% Sodium deoxycholate, 100 mM Sodium fluoride, 100 mM sodium pyrophosphate, 4 mM sodium orthovanadate, and 4 mM EDTA. Extracted protein amounts were quantified using Pierce BCA Protein Assay Kits (Thermo Fisher Scientific, Cat# 23227) and prepared in an SDS-containing buffer. Samples were subjected to SDS-polyacrylamide gel electrophoresis on 5%–20% SuperSep Ace (Wako, Cat# 197-15011). Primary antibodies included the following: p57 (SIGMA, Cat# P0357), Gapdh (Cloud Clone Crop., Cat# CAB932Hu22). Membranes were incubated consecutively with primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Promega, Cat #W4021, W4011), and signals were visualized with SuperSignal West Pico PLUS (Thermo Fisher Scientific, Cat# 34580) reagents and with a ChemiDoc imaging system (BioRad). Quantification of immunoblot intensity was performed using ImageJ. The p57 intensity was normalized with Gapdh.

### 4.4 | RNA-seq library preparation and analysis

Total RNA was extracted from cell lysates with the use of a PureLink RNA Mini Kit (Thermo Fisher Scientific, Cat# 12183025), and the quality of the purified RNA was assessed with a 2100 Bioanalyzer (Agilent). After mRNA selection with the use of a NEBNext Poly(A) mRNA Magnetic Isolation Module (New England BioLabs, Cat# E7490), libraries were prepared with the use of a NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England BioLabs, Cat# E7420). The cDNAs were sequenced with a NovaSeq 6000 system (Illumina). RNA-seq samples of each time point were prepared in triplicate.

### 4.5 | RNA-seq data analysis

Gene counts were acquired with the RHELIXA RNAseq pipeline on the NIG supercomputer (ROIS National Institute of Genetics). DESeq2 was applied to analyze differential expression. GSEA was performed with GSEA software 4.3.2.

### 4.6 | 4C-seq library preparation and analysis

The position of the mouse *Cdkn1c* promoter was obtained from the Eukaryotic Promoter Database (<https://epd.expasy.org/epd>). The region harboring the promoter sequence, flanked by MboI and NlaIII sites, was used as the viewpoint. For preparation of the ATRA-differentiated (day 8) sample, mESCs ( $3 \times 10^5$ ) were seeded on a gelatin-coated 100-mm dish and cultured in 2i medium for 2 days. The cells were then cultured for 2 days in cESC medium supplemented with 4  $\mu$ M ATRA and for an additional 4 days in cESC medium without ATRA, with daily medium renewal. For the preparation of the 2i (day 2) sample, cells were seeded as for the ATRA-differentiated sample and cultured in 2i medium for 8 days with daily medium renewal, to obtain a sufficient number of cells. On the final day of culture, cells for each sample type were collected and pooled separately to obtain  $2 \times 10^6$  cells for each library preparation. Library preparation for 4C-seq analysis was performed, as described (Krijger et al., 2020). In brief, the cells were fixed with 4% formaldehyde for 10 min and then lysed in a solution containing Nonidet P-40 and Triton X-100. The samples were digested with MboI (New England BioLabs, Cat# R0147) and subjected to ligation with T4 DNA ligase (TaKaRa, Cat# 2011). After reversal of cross-links with proteinase K (Roche, Cat# 03-115-801-001), DNA fragments were digested with NlaIII (New England BioLabs, Cat# R0125) and again subjected to ligation with T4 DNA ligase. The obtained circular 4C templates were subjected to inverse PCR with a primer pair targeting both ends of the viewpoint sequence in the outward direction and containing adapter sequences for indexing (5'-TACACGACGCTCTTCCGATCTAAGCTGGACAG-GACAAGC-3' as a read primer, and 5'-ACTGGAGTTCAGACGTGTGCTCTTCCGATCTCGCGCGCCCCCTC-GGAC-3' as a nonread primer). Finally, index PCR was performed on the inverse-PCR products with the use of NEBNext Multiplex Oligos for Illumina (New England BioLabs, Cat# E7335, E7500, E7710, E7730) to obtain the libraries. The libraries were sequenced with a NovaSeq 6000 system (Illumina). 4C-seq samples of each culture condition were prepared in triplicate.

#### 4.7 | 4C-seq data analysis

Peak calling for identification of cis-interactions was performed with the pipe4C pipeline, as described previously (Krijger et al., 2020). In brief, reads were “demultiplexed” according to the indexes, and qualified reads with the first restriction enzyme motif at the 5′ end were mapped to the mm10 reference genome. The restriction fragment ends were counted as read counts, and statistical analysis with peakC (Geeven et al., 2018) identified the regions with a higher contact frequency than expected.

#### 4.8 | Establishment of p57 gene enhancer-deficient mESCs

Gene-specific sgRNAs were designed to target sequences both upstream and downstream of the H3K4me1- and H3K27ac-enriched region in intron 2 of *Kcnq1* (5′-CTAGGCCACGCAGATATCA-3′ and 5′-GGGGTACTC TCCCGAACTGT-3′, respectively). The sgRNAs were designed with CRISPOR (<http://crispor.tefor.net/crispor.py>), and the corresponding DNA sequences were subcloned separately into the BbsI site of pX330 (Addgene). For preparation of a pX330 plasmid harboring both of the sgRNA sequences, the region from the U6 promoter to the RNA scaffold of one plasmid was amplified by PCR and then inserted into the XbaI site of the other plasmid with the use of the NEBuilder system (New England BioLabs, Cat# E2621). For establishment of mutant mESCs, stored cells were thawed and cultured in 2i medium for a few days before transfection. They were detached from the plate, replated, and transfected for 24 h with the pX330 plasmid harboring both sgRNA sequences with the use of the X-tremeGENE 9 reagent (Sigma, Cat# 6365809001). The cells were then subjected to selection with puromycin (1 µg/mL) for 30 h before reseeding and culture to allow the growth of colonies of a sufficient size. Colonies were picked up manually, and homozygous mutant clones were identified by genotyping PCR analysis with a mixture of two primer pairs, one of which was for detection of the mutant allele (P3 and P4: 5′-GTCTGTCCCAACTT TGCCATGTTC-3′ and 5′-GATCTTGAGGTCCTTAGAA GCAGCC-3′, respectively), and the other for that of the WT allele (P1 and P2: 5′-GACCCTCGTTCACACT CAGC-3′ and 5′-TGGCCAAATACAGTGCAAGCAG-3′, respectively). Clones determined to be homozygous mutants were stored at −80°C with the use of STEM-CELLBANKER (TaKaRa, Cat# CB047) for subsequent experiments.

#### 4.9 | CRISPRi for functional suppression of the putative p57 gene enhancer

The sgRNAs for CRISPRi were designed to target a sequence within the region deleted in mESCs as described above, as well as the EGFP gene as a control (#9, 5′-GTGGTTTGGGCTCCCGCGCT-3′; #16, 5′-AGGACACAT GCACCTTTCGG-3′; #30, 5′-TACATGCTGTGGACGG-GAGT-3′; #31, 5′-GGTGGAGAAGAAGTCACGCG-3′; and sgEGFP, 5′-CAACTACAAGACCCGCGCCG-3′). The corresponding DNA sequences were subcloned into the BsmBI site of pLV hU6-sgRNA hUb-dCas9-KRAB-T2a-Puro (Addgene). mESCs were subjected to CRISPRi by lentiviral infection as described below. HEK293T cells were transfected for 16 h with the constructed pLV vector as well as pCAG-HIVgp and pCMV-VSVG-RSG-Rev with the use of X-tremeGENE 9, after which the culture medium was changed to 2i medium and the cells were cultured for an additional 24 h. The culture supernatant was then collected and added to mESCs cultured in 2i medium. After infection for 2 days, the cells were subjected to selection with puromycin (1 µg/mL) for 30 h and then reseeded. The remaining cells were cultured until they reached the appropriate density and then stored with the use of STEM-CELLBANKER for subsequent experiments.

#### 4.10 | Statistical analysis

Data are presented as means ± SEM and were compared among groups with Welch's ANOVA test followed by Dunnett's test on GraphPad Prism version 10. A *p*-value of <.05 was considered statistically significant.

#### AUTHOR CONTRIBUTIONS

**Daisuke Koga:** Conceptualization; data curation; formal analysis; investigation; methodology; resources; validation; writing – original draft. **Shogo Nakayama:** Formal analysis; investigation; methodology; validation. **Tsunaki Higa:** Conceptualization; data curation; formal analysis; investigation; project administration; software; supervision; writing – original draft. **Keiichi I. Nakayama:** Conceptualization; funding acquisition; project administration; supervision; writing – original draft; writing – review and editing.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

## DATA AVAILABILITY STATEMENT

All sequence data have been deposited in the DDBJ Sequence Read Archive (DRA) database under accession codes PRJDB18234 for RNA-seq and PRJDB18233 for 4C-seq.

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