

GCNF-Dependent Activation of Cyclin D1 Expression via Repression of Mir302a During ESC Differentiation

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ABSTRACT

Cyclin D1 plays an important role in the regulation of cellular proliferation and its expression is activated during gastrulation in the mouse; however, it remains unknown how *cyclin D1* expression is regulated during early embryonic development. Here, we define the role of germ cell nuclear factor (GCNF) in the activation of cyclin D1 expression during embryonic stem cell (ESC) differentiation as a model of early development. During our study of GCNF knockout (*GCNF*^{-/-}) ESC, we discovered that loss of GCNF leads to the repression of cyclin D1 activation during ESC differentiation. This was determined to be an indirect effect of deregulation Mir302a, which is a cyclin D1 suppressor via binding to the 3'UTR of cyclin D1 mRNA. Moreover, we showed that *Mir302* is a target gene of GCNF that inhibits *Mir302* expression by binding to a DR0 element within its promoter. Inhibition of *Mir302a* using Mir302 inhibitor during differentiation of *GCNF*^{-/-} ESCs restored cyclin D1 expression. Similarly over-expression of GCNF during differentiation of *GCNF*^{-/-} ESCs rescued the inhibition of Mir302a expression and the activation of cyclin D1. These results reveal that GCNF plays a key role in regulating activation of cyclin D1 expression via inhibition of Mir302a. STEM CELLS 2014;32:1527–1537

INTRODUCTION

Cyclin D1 is a key cell cycle regulatory factor that facilitates progression through the G1/S checkpoint [1]. Its expression dramatically increases during gastrulation of mouse embryos or the differentiation of mouse embryonic stem cells (ESCs) [2, 3]. Cyclin D1 regulates the cell cycle and proliferation through interaction with CCNDBP1, retinoblastoma protein, cyclin-dependent kinase (CDK)–4, CDK-6, and proliferating cell nuclear antigen (PCNA) [4–7]. Cyclin D1 was found not only in proliferating cells (e.g., kidney and intestine) but also in the nondividing cells (e.g., mature neurons of the adult brain), it is especially highly expressed in neurons of the developing embryonic nervous system [8, 9]. *Cyclin D1*-deficient mice are smaller in size, underdeveloped, and display hypoplastic retinas, hypoplastic mammary glands, and developmental neurological abnormalities [10, 11]. Mouse ESCs actively proliferate with a mitotic S phase that is largely predominant and a G1 phase that lasts no longer than 1.5 hours consistent with the absence of a G1/S checkpoint. ESCs use a D-cyclin-independent cell cycle machinery to maintain both proliferation and inhibition of differentiation in response to

phosphatidyl inositol-3 kinase signaling in undifferentiated ESCs [12–14]. Early embryonic pluripotent cells and derivative ESCs express low cyclin D1 levels, no cyclin D2, nor cyclin D3 [2]. Clearly there exists a switch that regulates the transition from cyclin D-independent proliferation in undifferentiated ESCs to cyclin D-dependent cell cycle as ESCs start to differentiate. A crucial question is what mechanism underlies this switch and what factors regulate it during ESC differentiation.

Increasing evidence shows that microRNAs play a pivotal role in post-transcriptional regulation by inhibiting gene expression through selective binding to complementary mRNA 3'UTR sequences [15]. Recently, microRNAs have been shown to regulate the cell cycle G1/S transition and promote rapid proliferation in ESCs [16]. Mir302a was reported to inhibit cyclin D1 expression by binding the cyclin D1 3'UTR in human ESCs [3] and to regulate cancer cell [17] or somatic cell reprogramming [18] into a pluripotent state. These data imply that Mir302a probably plays an important role in regulating activation of cyclin D1 expression during ESC differentiation.

Germ cell nuclear factor (GCNF; NR6a1), a transcriptional repressor and an orphan member of the nuclear receptor gene family, is

required for the repression and silencing of pluripotency genes such as *Oct4* and *Nanog* in vitro and in vivo [19, 20]. Loss of GCNF function in *GCNF*^{-/-} mice results in embryonic lethality by E10.5 [21]. Importantly, there is loss of normal repression of the *Oct4* gene in somatic cells after gastrulation, where it is generally silenced. Likewise, *GCNF*^{-/-} ESCs maintain *Oct4* expression during differentiation upon retinoic acid (RA) treatment, which impedes full differentiation of these cells [22].

Here, we report that GCNF is required to activate cyclin D1 expression during ESCs differentiation and mouse embryonic development. However, GCNF is a transcriptional repressor [20, 23, 24] and its regulation of cyclin D1 expression was shown to be indirect. GCNF suppresses Mir302a expression during mESC differentiation in turn permitting induction of cyclin D1 expression. The repression of Mir302a expression by GCNF is direct through binding to an element in its promoter. In *GCNF*^{-/-} ESCs Mir302a levels are elevated leading to direct suppression of cyclin D1 expression during differentiation. Here, we demonstrate that GCNF's repression of *Mir302a* expression is the key event that facilitates activation of cyclin D1 expression during ESC differentiation.

MATERIALS AND METHODS

ESC Lines and Differentiation and Embryos

Wild-type and *GCNF*^{-/-} ESCs were produced from 3.5 d.p.c. embryos, and were maintained in an undifferentiated state on gelatin-coated dishes as described previously [22]. E8.5 embryos of wt and *GCNF*^{-/-} genotype were harvested from E8.5 pregnant mice. ESCs were maintained in Dulbecco's modified Eagle's medium (DMEM) media (Life Technologies, Grand Island, NY, <http://www.lifetechnologies.com>) with 15% fetal bovine serum (FBS) (Hyclone, Thermo Fisher Scientific, Waltham, MA, <http://www.thermoscientific.com>), 1 × Minimum essential medium (MEM) nonessential amino acids (Life Technologies, Grand Island, NY, <http://www.lifetechnologies.com>), 2 mM L-glutamine (Life Technologies, Grand Island, NY, <http://www.lifetechnologies.com>), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, <http://www.sigmaaldrich.com>), and 1,000 U/ml Leukemia inhibitory factor (LIF) (Chemicon (Merck Millipore), Temecula, CA, <http://www.emdmillipore.com>). ESCs were induced to differentiate by treatment with 1 μM RA (Sigma-Aldrich, St. Louis, MO, <http://www.sigmaaldrich.com>) and LIF removal on gelatin-coated dishes or by embryoid body (EBs) formation in Petri dishes.

Teratoma Formation

Teratoma formation was used to evaluate proliferation in vivo, briefly, 1 × 10⁶ wt and *GCNF*^{-/-} ESCs were injected into the rear leg muscle of 4–6-week-old severe combined immunodeficient (SCID) mice. Four weeks later, tumors were surgically dissected post mortem from the euthanized mice.

Immunohistochemistry

E8.5 embryos were fixed in 4% paraformaldehyde and embedded in paraffin. Sections of 5 μm were deparaffinized using xylene and ethanol, and then rehydrated in water for 1 minute. The sections were blocked with 10% horse serum and 1% bovine serum albumin (BSA). Primary antibodies against cyclin D1 (Thermo Fisher Scientific, Waltham, MA, <http://www.thermoscientific.com>) were diluted 1:200 and normal

rabbit IgG (Santa Cruz, Santa Cruz, CA, <http://www.scbt.com>) was used as control. Secondary antibody Texas-red-labeled goat anti-rabbit antibodies (Vector Laboratories, Burlingame, CA, <https://www.vectorlabs.com>) were diluted 1:500.

Analysis of Cell Cycle by Flow Cytometry

wt and *GCNF*^{-/-} cells were trypsinized, washed twice with phosphate buffered saline (PBS), resuspended in 300 μl PBS, and then added 5 ml of cold 70% ethanol to fix cells overnight at 4°C. After fixation, the cells were washed once with PBS; 1 ml of staining solution of 10 μg/ml propidium iodide (Sigma-Aldrich, St. Louis, MO, <http://www.sigmaaldrich.com>) and 100 μg/ml DNase-free RNase (Sigma-Aldrich, St. Louis, MO, <http://www.sigmaaldrich.com>) were added to each sample (2 × 10⁶ cells) and the cells were incubated in the dark at 37°C for 20 minutes. Cell cycles were analyzed using a BDIS FACScan.

Isolation and Analysis of mRNAs and microRNAs

Total RNAs were isolated using TRIzol reagent (Invitrogen, Life Technologies, Grand Island, NY, <http://www.lifetechnologies.com>) according to the manufacturer's instructions. RNA was analyzed using regular reverse transcription PCR (RT-PCR) or quantitative RT-PCR (qRT-PCR) with sybr green Q-PCR reagent (Qiagen, Valencia, CA, www.qiagen.com). RNA samples were reverse transcribed to cDNA using SuperScript III First-Strand Synthesis System (Invitrogen, Life Technologies, Grand Island, NY, <http://www.lifetechnologies.com>). PCR primers used in this article were listed as Supporting Information Table S1. Mir302a was analyzed with hsa-miR302 TaqMan MicroRNA Assay, and U6 snRNA as control using Taqman reagents (Invitrogen, Life Technologies, Grand Island, NY, <http://www.lifetechnologies.com>). Northern blot was also used to analyze the Mir302 (probe: 5'TCACAAAACATGGAAGCAC TTA3') expression of wt and *GCNF*^{-/-} ESCs. Briefly, 30 mg of total RNA was resolved in a 15% denaturing polyacrylamide gel containing 7 M urea in 0.5× Tris-Borate-EDTA (TBE) buffer system and transferred onto Zeta-Probe membrane (Bio-Rad, Hercules, CA, <http://www.bio-rad.com>) in 0.5× TBE. Mir302 probe oligonucleotides were radioactively labeled with ³²P-ATP (MP Biomedicals, Santa Ana, CA, <http://www.mpbio.com>) and T4 kinase (Invitrogen, Life Technologies, Grand Island, NY, <http://www.lifetechnologies.com>). Ultraviolet light-cross linked membranes were hybridized with radioactively labeled DNA probe at 45°C in Quickhyb solution (Stratagene, Agilent Technologies, La Jolla, CA, <http://www.genomics.agilent.com>) and washed with 2× saline-sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS). The radioactive signals were detected by photographic film.

Western Analysis

Whole cell lysates were obtained by treatment with lysis buffer (0.1% SDS, 0.5% triton-100, 20 mM Tris pH 7.5, and 150 mM NaCl) and were subjected to Western blot analyses. Antibodies against Oct4 (Santa Cruz, Santa Cruz, CA, <http://www.scbt.com>) and GCNF were used at a 1:1,000 dilution; cyclin D1 antibodies were used at a 1:1,000 dilution. β-Actin antibodies (Sigma-Aldrich, St. Louis, MO, <http://www.sigmaaldrich.com>) were used at a 1:5,000 dilution. All antibodies were diluted in tris-buffered saline (TBS) with 5% fat-free dry milk. The secondary antibodies, goat anti-mouse IgG-horseradish peroxidase (HRP) (Santa Cruz), and goat anti-rabbit IgG-HRP (Santa Cruz, Santa Cruz, CA, <http://www.scbt.com>) were used at a 1:2,000 dilution.

HRP activity was detected using chemiluminescence with Pierce ECL Western Blotting Substrate kit (Thermo Fisher Scientific, Waltham, MA, <http://www.thermoscientific.com>). The emitted light was detected by photographic film.

Chromatin Immunoprecipitation Analysis

Sonicated DNA samples of undifferentiated and differentiated ESCs at different time points were used for chromatin immunoprecipitation (ChIP) assays as described previously [25]. The antibodies that were used for the ChIP assays were against GCNF. qRT-PCR was carried out using SYBR Green FastMix (Qiagen, Valencia, CA, www.qiagen.com) with an ABI 7900HT real-time PCR machine. The following primer sets spanning the Mir302a promoter were used: Mir302a forward: 5'CTGGCTGCTCG GAACTCACT3', reverse: 5'TCATGCCTTTGATCCAGCACT3'.

Electrophoretic Mobility Shift Assays

Nuclear extracts were extracted with binding buffer (25 mM HEPES, pH 7.9, 150 mM KCl, 0.4 mM EDTA, 2 mM dithiothreitol, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1_x protease inhibitor cocktail [Roche, Indianapolis, IN, www.rocheusa.com]). Nuclear extracts were prepared from undifferentiated and differentiated wt and *GCNF*^{-/-} ESCs at different time points of RA treatment, 1, 2, 3, 4, 5, and 6 days. Oligonucleotides that contained complementary sequences (Forward: 5'GTAGACCAGGCTGGCCTTGAAC TCAGAAAT3'; Reverse: 5'CGGATTTCTGAGTTCAAGGCCAGCCTGG T3') to the Mir302a putative GCNF binding site were labeled with P[32] isotope and were mixed with nuclear extracts before samples were loaded. GCNF antibodies were added to nuclear extracts in supershift experiments. Electrophoretic mobility shift assays (EMSA) were performed as described by Garner and Revzin [26].

Luciferase Reporter Assays

pMIR-report luciferase plasmid (Ambion, Life Technologies, Grand Island, NY, <http://www.lifetechnologies.com>) was used to analyze the effect of Mir302a on inhibiting cyclin D1 by binding the 3'UTR. The synthesized 3'UTR oligonucleotides of cyclin D1 with Mir302 binding site were cloned into the multiple cloning site of pMIR-reporter luciferase plasmid using Sca1 and Hind III enzymes. The synthesized 3'UTR oligonucleotides of cyclin D1 with mutant Mir302 binding site were also cloned as a control (Fig. 5A, Supporting Information Fig. S3). pGL3 plasmid was used to analyze the effect of GCNF on inhibiting Mir302a by binding the DR0 within Mir302a promoter. The DNA sequences of Mir302a promoter were amplified using PCR using ESC DNA. The primer sets were listed below:

Forward1: 5'TCGGTACCAAGAATAAAGCCTTA3';

Forward2: 5' TCGGTACTCTGGAAGAGCAGTCA3';

Reverse: 5'CCCTCGAGTCTGTTCTGCTCG3'; Forward 1 primer was used to amplify the promoter with the GCNF binding DR0 site; forward 2 primer was used to amplify the promoter without the GCNF binding DR0 site. The amplified promoter amplicon was cloned into the pGL3 plasmid using Kpn1 and Xho1 enzyme sites. The empty plasmid was used as a control. Transfected cells were lysed in 500 μ l of passive lysis buffer (Promega, Madison, WI, <http://www.promega.com>) and assayed with a dual luciferase assay as directed by the manufacturers' instructions. The luciferase activities were expressed as relative luciferase/Renilla activities, normalized to those of control transfections in each case.

Mir302 Inhibition

The miRNA 302 inhibitor (Dharmacon, Thermo Fisher Scientific, Waltham, MA, <http://www.thermoscientific.com>) and

miRIDIAN microRNA Inhibitor Negative Control #1 (Mir302NC) (Dharmacon, Thermo Fisher Scientific, Waltham, MA, <http://www.thermoscientific.com>) were transfected into 7.5×10^5 *GCNF*^{-/-} ESCs in each well of six-well plates, respectively, using Lipofectamine 2000 (Invitrogen, Life Technologies, Grand Island, NY, <http://www.lifetechnologies.com>) reagent according to the manufacturer's instructions. After transfection, the cells were treated with RA to induce ESC differentiation. Three days later, the protein samples were collected for Western blot analysis.

Plasmid Construction and Transfection

A full-length mouse GCNF complementary DNA (cDNA) was obtained from reverse transcribed PCR products generated from ESC mRNA. The resulting cDNA was cloned into the Gateway/Topo TA vector (Invitrogen, Life Technologies, Grand Island, NY, <http://www.lifetechnologies.com>), and was then recombined into the destination retroviral plasmid with the mscv promoter by Gateway LR Clonase (Invitrogen, Life Technologies, Grand Island, NY, <http://www.lifetechnologies.com>). For packaging the virus, 1.5×10^6 BOSC 23 (BOSC, derived from the Ad5-transformed human embryonic kidney 293 cell line) cells were plated in six-well of plates (10% FBS DMEM), and were cultured overnight. Mscv-GCNF, mscv-green fluorescent protein (GFP) with packaging plasmid pDuo were introduced into each 6 cm plate with BOSC cells [27], respectively, using Fugene 6 transfection reagent (Roche, Indianapolis, IN, www.rocheusa.com) according to the manufacturers' recommendations. After 24 hours of transduction, virus-containing supernatants derived from these BOSC cell cultures were filtered through a 0.45 mm cellulose acetate filter and supplemented with 4 mg/ml polybrene. Target cells (3×10^5) were seeded into 24-well plates that were incubated with 2.5 ml of the virus/polybrene-containing supernatants overnight and infected twice. After 24 hours of infection, the media were replaced with 2.5 ml fresh ESC medium. The transfected cells were selected for 2 weeks with 1 μ g/ml of puromycin after 3 days of transfection. The selected cells were used for the described analysis.

Statistics Analysis

All experiments were replicated three times. All data were presented as mean \pm SD. Student's *t* test was performed to determine the differences among grouped data. * indicates statistically significance with *p* < .05.

RESULTS

Loss of GCNF Alters Gene Expression and Proliferation in ESCs

GCNF is an indispensable evolutionarily conserved transcription factor in embryonic development [28]. Inactivation of the GCNF gene leads to changes in the morphological features of ESC colonies. The morphology of the *GCNF* knockout (*GCNF*^{-/-}) ESC colonies compared to wild-type (wt) ESCs lacks multiple layers, typically characteristic of mouse ESC colonies, rather they form a single layer on gelatin-coated plates; appearing like human ESCs in nature (Fig. 1A). After the ESCs were induced to differentiate with RA, the *GCNF*^{-/-} ESCs still maintained an undifferentiated morphology: round and small, compact nucleus, with a high nucleus-to-cytoplasm volume

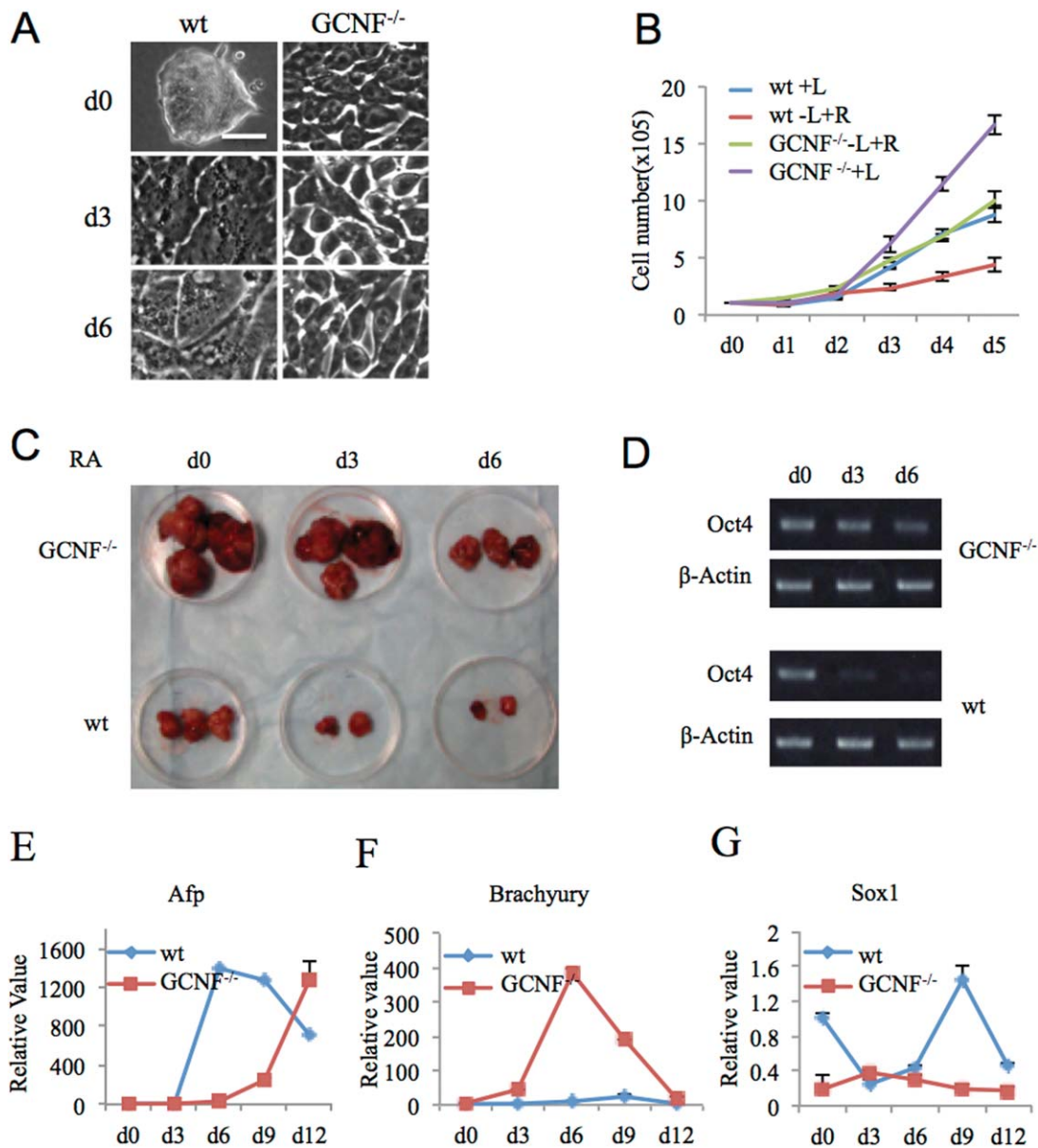


Figure 1. GCNF promotes cell proliferation and loss of GCNF expression alters embryonic stem cell (ESC) colony morphology. **(A):** Wt and GCNF^{-/-} ESCs were plated on gelatin-coated dishes, the undifferentiated ESCs were cultured in ESC media in order to maintain the undifferentiated state; the differentiated ESCs were induced by RA and LIF removal. The morphology of wt and GCNF^{-/-} ESCs was observed under microscopy in undifferentiated (d0) state and RA induced differentiated state at d3 and d6. Scale bar = 20 μ m. **(B):** Cells were plated on gelatin-coated plates and were counted daily for 5 days. Undifferentiated cells (d0) were cultured in ESC media and differentiated cells were cultured in the media without LIF and with RA. **(C):** 1×10^6 wt and GCNF^{-/-} ESCs were injected in severe combined immuno-deficient mice with cells at different differentiation time points: d0, d3, and d6, and teratoma were harvested at 28 days after injection. **(D):** RT-PCR results of Oct4 expression in the wt and GCNF^{-/-} ESCs at related different time points: d0, d3, and d6. **(E):** Undifferentiated wt and GCNF^{-/-} ESC RNAs were cultured on gelatin-coated dishes in ESC media; differentiated ESCs were cultured on Petri dishes in differentiated ESC media and then the cells were aggregated into embryoid bodies (EBs). RNA samples were isolated from undifferentiated cells (d0) and differentiated cells at d3, d6, d9, and d12 of EB formation, respectively. Afp expression was assayed by Q-RT-PCR. **(F):** Brachyury expression was analyzed by Q-RT-PCR. **(G):** Sox1 was tested by Q-RT-PCR. Abbreviations: GCNF, germ cell nuclear factor; RA, retinoic acid.

ratio, even at day 6 of differentiation. In contrast, the morphology of RA-treated wt ESCs possesses characteristics of a differentiated cell, they are larger and irregular in shape and they have a lower nucleus-to-cytoplasm volume ratio (Fig. 1A).

GCNF^{-/-} ESCs also showed a faster proliferation rate than wt ESCs in both undifferentiated and differentiated states in

vitro (Fig. 1B). In vivo proliferation of both ESC types was also evaluated by injection of undifferentiated and differentiated (d3 and d6) wt or GCNF^{-/-} ESCs into the SCID mice, respectively. Four weeks later, the size of the teratomas from GCNF^{-/-} ESCs was found to be significantly larger than those derived from wt ESCs in all three corresponding samples (Fig. 1C). In general, teratoma formation in undifferentiated ESCs

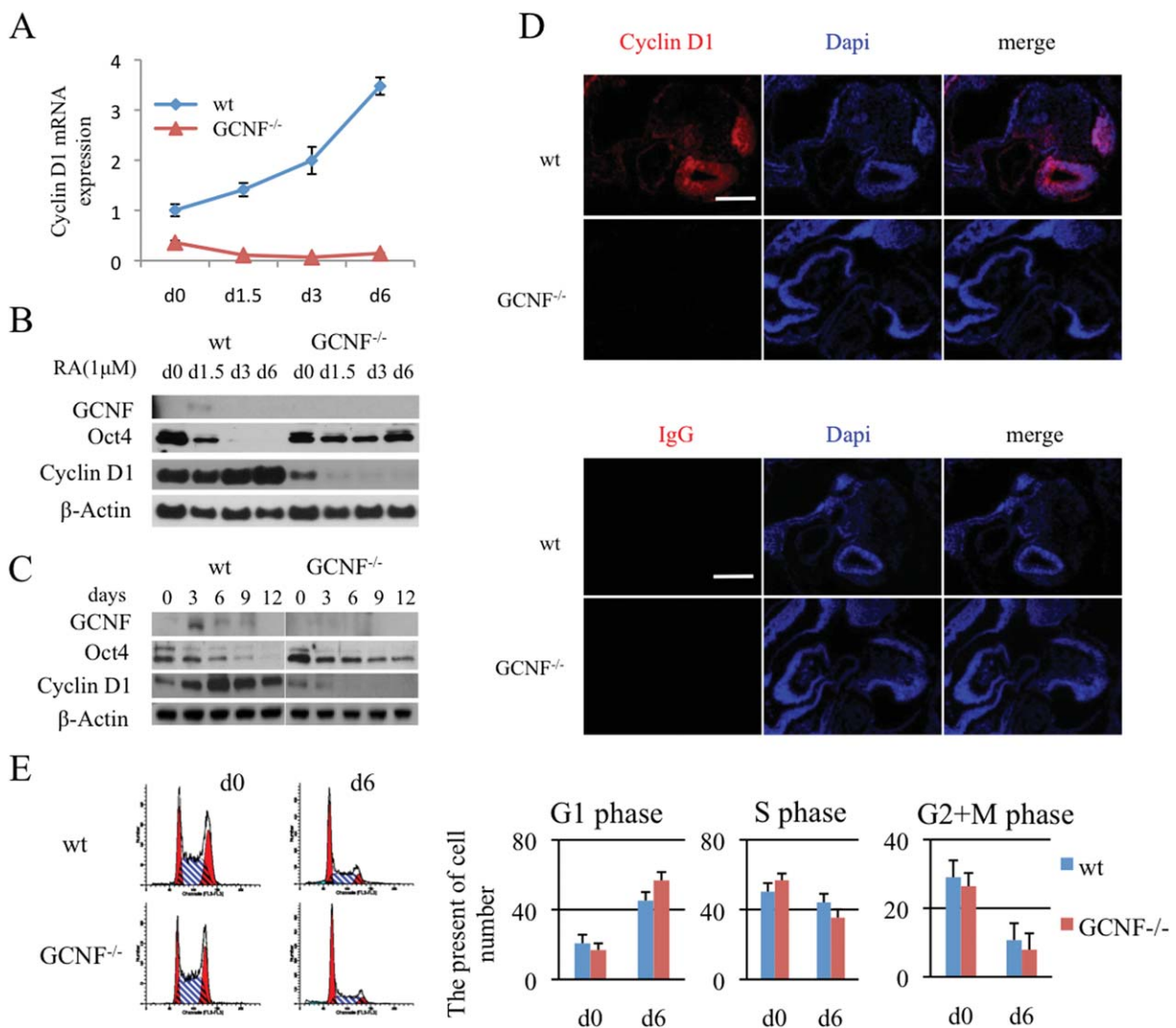


Figure 2. Loss of *GCNF* expression fails to activate cyclin D1 expression. **(A)**: mRNA was isolated from wt embryonic stem cells (ESCs) and *GCNF*^{-/-} ESCs in undifferentiated (d0) and differentiated states (d1.5, d3, and d6) induced with RA, and cyclin D1 expression was analyzed by Q-RT-PCR. Results were normalized to GAPDH expression. Error bars represent \pm SD. **(B)**: Whole cell lysates were analyzed by Western blot using anti-*GCNF*, anti-*Oct4*, and anti-cyclin D1 antibodies. Anti- β -actin was used as a loading control. **(C)**: Whole cell lysates from embryoid body samples were analyzed by Western blot using anti-*GCNF*, anti-*Oct4*, and anti-cyclin D1 antibodies. Anti- β -actin was used as a loading control. **(D)**: E8.5 embryos, wt and *GCNF*^{-/-}, were stained with anti-cyclin D1 and fluorescently labeled secondary antibody. Dapi was used to counter stain cell nuclei; IgG staining was used as a negative control. Scale bar = 500 μ m. **(E)**: The cell cycle analysis of wt ESCs and *GCNF*^{-/-} ESCs in undifferentiated and differentiated (at day 6) state by fluorescence-activated cell sorting. Abbreviations: *GCNF*, germ cell nuclear factor; RA, retinoic acid.

was the largest, and teratomas from day 6 differentiated ESCs were the smallest. The size of teratomas from *GCNF*^{-/-} ESCs was consistently larger relative to those derived from wt cells (Fig. 1C) correlating with the maintenance of *Oct4* gene expression in differentiated *GCNF*^{-/-} ESCs and the downregulation of *Oct4* in wt ESCs (Fig. 1D). Maintenance of an undifferentiated morphology is most probably caused by the expression of relatively high levels of pluripotency genes in differentiated *GCNF*^{-/-} ESCs [23]. In addition, inactivation of *GCNF* did not affect the overall ability to form EBs (Supporting Information Fig. S1).

The expression pattern of lineage marker genes was altered between wt EBs and *GCNF*^{-/-} EBs. Alpha-fetoprotein (*Afp*), a marker of endoderm (Fig. 1E), increased from day 3 of differentiation during wt EB formation. However, delayed induction of (*Afp*) expression was observed during *GCNF*^{-/-}

EB differentiation. *Brachyury*, a marker of mesoderm, was induced more quickly and earlier during *GCNF*^{-/-} EB differentiation than wt EB differentiation (Fig. 1F). *Sox1*, a marker of neuroectoderm, decreased initially during wt EB differentiation, and then increased to a maximum at day 9. In contrast, *Sox1* expression remained at low levels during *GCNF*^{-/-} EB differentiation (Fig. 1G). Thus, loss of *GCNF* has changed the typical characteristics of ESCs, including more rapid proliferation, which prompted us to analyze expression of cell cycle regulatory factors.

Failure to Activate Cyclin D1 Expression During *GCNF*^{-/-} ESC Differentiation and in *GCNF*^{-/-} Embryos

Analyses have shown that cyclin D1 protein is expressed at low levels in mouse blastocysts prior to gastrulation (E6.5) [2]

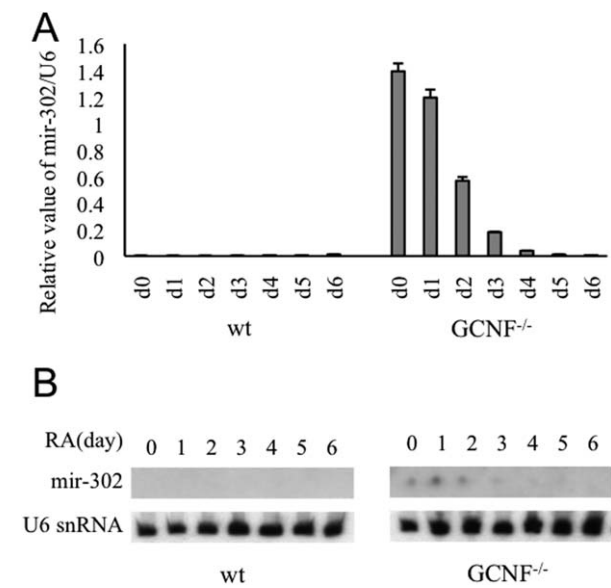


Figure 3. Mir302a expression in wt and *GCNF*^{-/-} embryonic stem cells (ESCs). **(A):** Mir302 expression was analyzed by Q-RT-PCR in wt and *GCNF*^{-/-} ESCs in the undifferentiated state (d0) and in a differentiated state (RA treatment for d1, d2, d3, d4, d5, and d6). U6 snRNA was used as a control. **(B):** Mir302 expression was analyzed by Northern blot in wt and *GCNF*^{-/-} ESCs in the undifferentiated state (d0) and in a differentiated state (RA treatment for d1, d2, d3, d4, d5, and d6). U6 snRNA was used as a loading control. Abbreviations: GCNF, germ cell nuclear factor; RA, retinoic acid.

and in undifferentiated mouse ESCs consistent with the absence of a G1/S check point [12]. The transition from cyclin D independence to cyclin D dependence is an intrinsic hallmark of pluripotent cell differentiation during early embryonic development [29]. To investigate GCNF's role in regulating cyclin D1 expression, we took advantage of a model ESC system that we derived from blastocyst outgrowths. Wt and *GCNF*^{-/-} ESCs were induced to differentiate with RA treatment after LIF removal. RNA and protein were isolated from undifferentiated ESCs (at day 0) (d0) and differentiated ESCs at day 1.5 (d1.5), d3, and d6. Quantitative real-time polymerase chain reaction (qRT-PCR) of cyclin D1 mRNA demonstrated that cyclin D1 was expressed at low levels in undifferentiated ESCs and quickly increased after differentiation in wt ESCs (Fig. 2A). Western blot of whole cell lysates using anti-GCNF, Oct4 and cyclin D1 antibody showed that Oct4 expression is maintained in *GCNF*^{-/-} ESCs during differentiation compared to the rapid decrease in wt ESCs during differentiation (Fig. 2B) [22]; cyclin D1 was expressed at low levels in undifferentiated ESCs and was quickly activated after RA induced differentiation in wt ESCs (Fig. 2B). In contrast, cyclin D1 expression decreased rather than increased at both the mRNA and protein levels after differentiation of *GCNF*^{-/-} ESCs. Although cyclin D1 was expressed in undifferentiated *GCNF*^{-/-} ESCs, cyclin D1 mRNA and protein levels are lower than that of wt ESCs and were not induced upon ESC differentiation with RA treatment (Fig. 2A, 2B).

To further investigate the dynamic change of cyclin D1 expression during ESC differentiation, we induced *GCNF*^{-/-} ESCs to differentiate by EB formation without RA treatment. Western blot of whole cell lysates demonstrated that Oct4

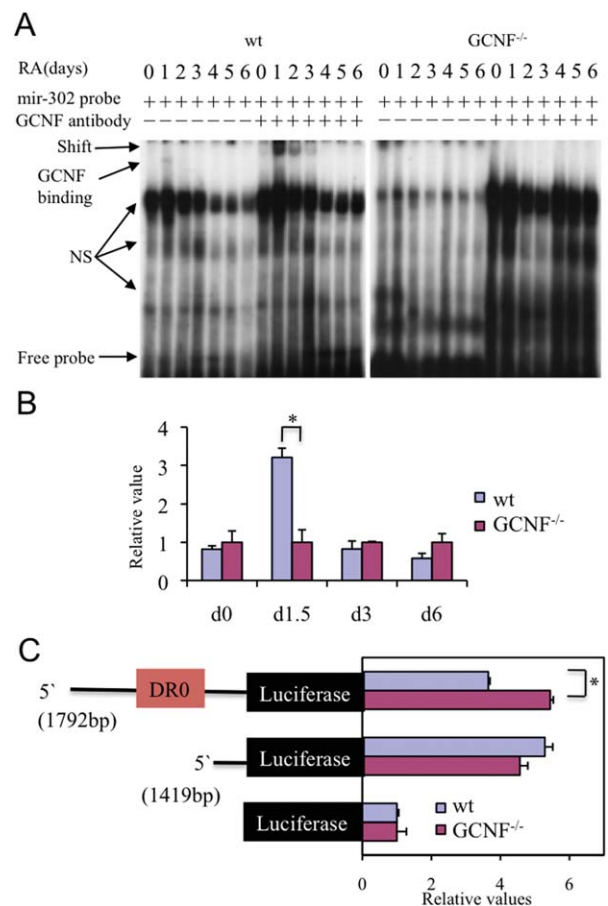


Figure 4. GCNF directly inhibits Mir302 expression by binding to a DR0 element in the promoter of *Mir302*. **(A):** Electrophoretic mobility shift assay analysis of GCNF binding to the DR0 within the *Mir302* promoter. An oligonucleotide probe containing the DR0 element was labeled and used to detect GCNF DNA binding in vitro. Anti-GCNF antibodies were used to detect if the retarded GCNF protein. **(B):** Chromatin immunoprecipitation analysis of GCNF binding to the DR0 within the *Mir302* promoter was used to assess binding in vivo (*, $p < .05$). **(C):** Luciferase reporter assays were used to analyze the GCNF-dependent inhibition of *Mir302* transcription (*, $p < .05$). Abbreviations: GCNF, germ cell nuclear factor; RA, retinoic acid.

expression is maintained during *GCNF*^{-/-} EB formation. Surprisingly, GCNF was still transiently induced in wt ESCs during EB formation, similar to RA induced differentiation (Fig. 2C); cyclin D1 was not activated till day 12 of differentiation in *GCNF*^{-/-} EBs, while cyclin D1 expression was activated in wt differentiated EBs (Fig. 2C). These results suggested that GCNF plays a crucial role in regulating the induction of cyclin D1 expression during ESC differentiation.

To determine whether these in vitro results in ESCs were corroborated in vivo, we analyzed cyclin D1 expression in wt and *GCNF*^{-/-} embryos. Cyclin D1 expression was analyzed during mouse embryonic development at embryonic day 8.5 (E8.5) using immunofluorescence staining; rabbit anti-mouse cyclin D1 antibody was used in experiment and normal rabbit IgG was used as negative control. Similar results were observed in vivo during mouse embryonic development as during ESC differentiation. Cyclin D1 protein was detected in most tissues of wt mouse E8.5 embryos, with high levels detected in the neural epithelium, which is highly

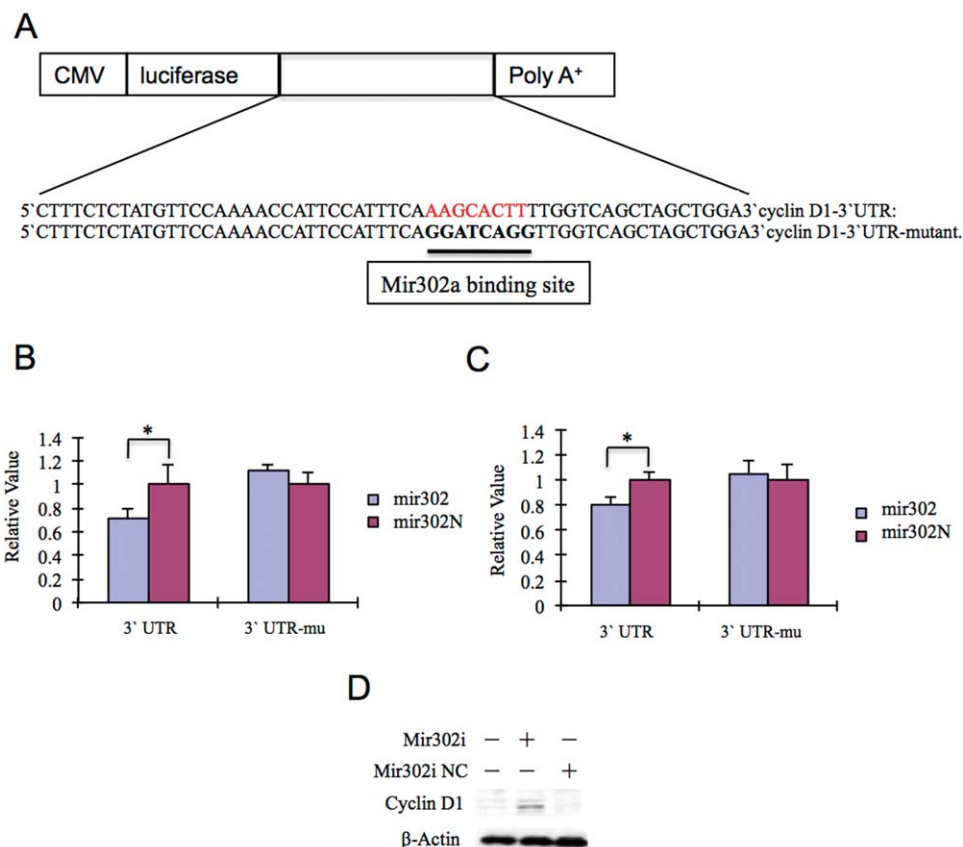


Figure 5. Inhibition of Mir302a expression rescued cyclin D1 expression during *GCNF*^{-/-} embryonic stem cell (ESC) differentiation. **(A):** Luciferase reporter plasmids were constructed with pMiR-reporter vector into which the cyclin D1 3'UTR was inserted, containing the Mir302 binding site or a mutant cyclin D1 3'UTR as a control. **(B):** Relative luciferase activities were analyzed in P19 cells after a Mir302 mimic was cotransfected with the normal and mutant cyclin D1 3'UTR reporters (*, *p* < .05). **(C):** Relative luciferase activities were analyzed in 293 cells after a Mir302 mimic was cotransfected with the normal and mutant cyclin D1 3'UTR reporters (*, *p* < .05). **(D):** Mir302 inhibitor (mir302i) and a mir302 negative control (mir302 NC) were transfected into *GCNF*^{-/-} ESCs, which were then induced to differentiate with retinoic acid for 3 days. Western blot of cyclin D1 expression was performed with whole cell isolates.

proliferative. In contrast, no cyclin D1 protein expression was detected in E8.5 *GCNF*^{-/-} embryos (Fig. 2D). The loss of cyclin D1 expression likely contributes to the phenotype in the *GCNF*^{-/-} embryo, which is lethal before E10.5 and displays an open neural tube and posterior truncation [21].

Proliferation is controlled by the cell cycle, thus we investigated the effect of GCNF on cell cycle regulation. Using fluorescence-activated cell sorting analysis to evaluate the cell cycle profiles of propidium iodide stained cells, we were able to determine differences between wt and *GCNF*^{-/-} ESCs (Fig. 2E). The cell number of G1 phase of undifferentiated *GCNF*^{-/-} cells was lower than wt undifferentiated ESC and increased during cell differentiation, more than observed in wt ESCs at day 6 (Fig. 2E). In contrast, the number of S phase *GCNF*^{-/-} ESCs was higher than WT ESCs in undifferentiated state, and then decreased to a lower level than that of WT ESCs after differentiation at day 6 (Fig. 2E). G2+M phase cells decreased both in wt and in *GCNF*^{-/-} ESCs (Fig. 2E). These results indicate that GCNF regulates the cell cycle during ESC differentiation.

Mir302a Expression Dramatically Increased in *GCNF*^{-/-} ESCs

This is the first demonstration that GCNF plays a key role in regulating the expression of cyclin D1 during early embryonic

development, which contrasts with its demonstrated role as a transcriptional repressor during ESC differentiation [19, 20]. DR0 elements to which GCNF binds were not found in the *cyclin D1* promoter, this in conjunction with the fact that no transcriptional activation function has been demonstrated for GCNF led us to investigate indirect mechanisms that would bridge its transcriptional repressor function and gene activation, such as miRNA regulation. Recent studies have shown that introducing exogenous pre-Mir302a into HeLa cells inhibits cyclin D1 expression; Mir302a is also expressed at low levels in E3.5 wt embryos [3]. Previous array analysis of microRNAs expression during ESC differentiation demonstrated that Mir302 was highly upregulated in *GCNF*^{-/-} ESCs relative to wt ESCs [30]. To validate if Mir302a was highly expressed in *GCNF*^{-/-} ESCs, qRT-PCR was used to detect Mir302a expression in wt and *GCNF*^{-/-} ESCs. High level expression of Mir302a was found in undifferentiated and early differentiating *GCNF*^{-/-} ESCs (Fig. 3A) compared to the low levels of Mir302a in both undifferentiated and early differentiating wt ESCs (Fig. 3A). We used Northern blot to further confirm the differential Mir302a expression pattern. The results showed that Mir302a was expressed at high levels in undifferentiated and early differentiating *GCNF*^{-/-} ESCs; no Mir302 was detected in wt ESCs (Fig. 3B). Mir302a expression at low

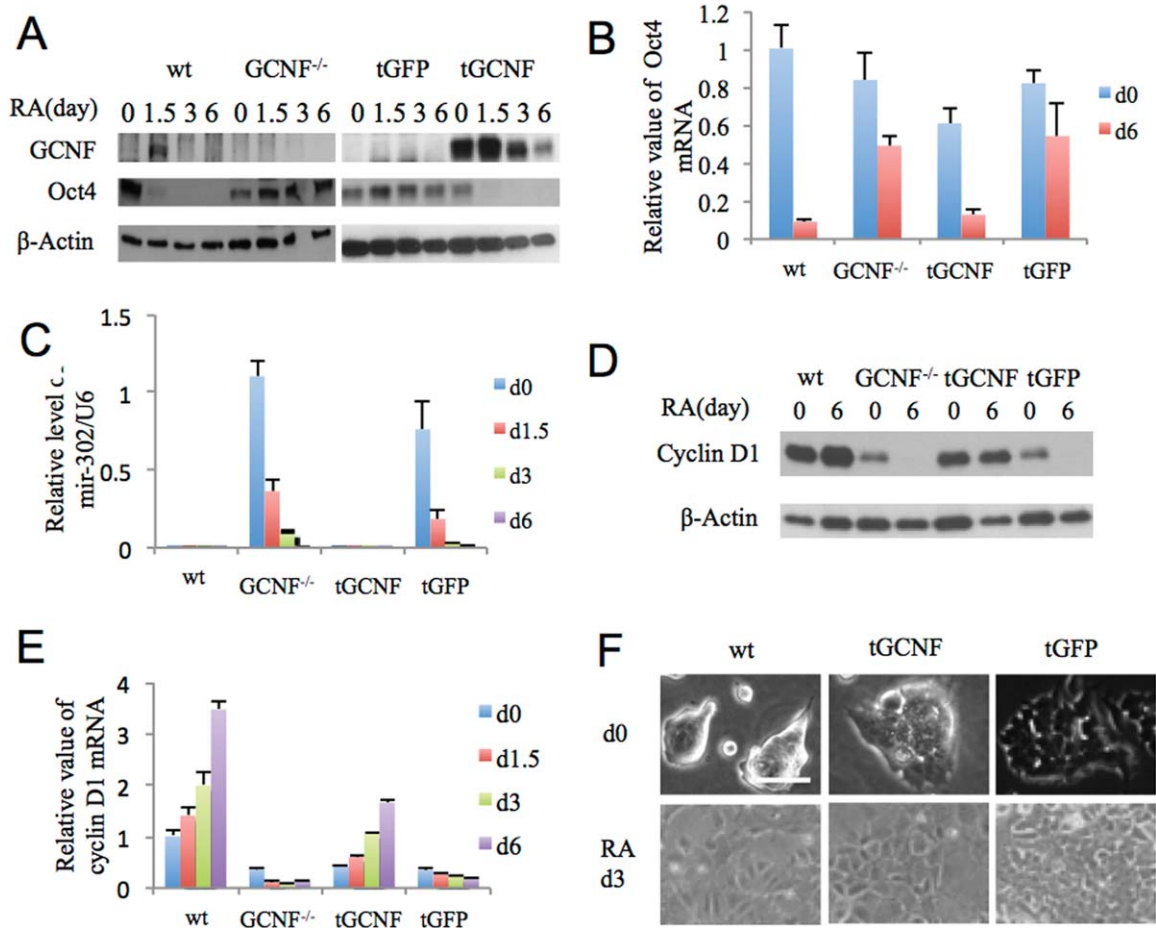


Figure 6. Over-expression of GCNF rescues activation of cyclin D1 expression by repression of Mir302 expression. **(A):** GCNF expressing retroviruses and GFP control retroviruses were transfected in to $GCNF^{-/-}$ embryonic stem cells (ESCs). Protein levels of GCNF and Oct4 were analyzed by Western blot in wt ESCs, $GCNF^{-/-}$ ESCs, GFP expressing $GCNF^{-/-}$ ESCs (tGFP), and GCNF transfected $GCNF^{-/-}$ ESCs (tGCNF) during differentiation. Anti- β -actin was used as a loading control. **(B):** The relative mRNA levels of Oct4 to GAPDH were analyzed by qRT-PCR in wt ESCs, $GCNF^{-/-}$ ESCs, GCNF transfected $GCNF^{-/-}$ ESCs, and GFP transfected $GCNF^{-/-}$ ESCs during differentiation. **(C):** Mir302 expression was analyzed in wt ESCs, $GCNF^{-/-}$ ESCs, GCNF transfected $GCNF^{-/-}$ ESCs, and GFP transfected $GCNF^{-/-}$ ESCs by qRT-PCR during differentiation. **(D):** Cyclin D1 protein levels were analyzed by Western blot in wt ESCs, $GCNF^{-/-}$ ESCs, GCNF transfected $GCNF^{-/-}$ ESCs, and GFP transfected $GCNF^{-/-}$ ESCs at d0 (undifferentiated) and at d6 (differentiated). **(E):** Cyclin D1 mRNA levels were analyzed by qRT-PCR in wt ESCs, $GCNF^{-/-}$ ESCs, GCNF transfected $GCNF^{-/-}$ ESCs, and GFP transfected $GCNF^{-/-}$ ESCs at d0, d1.3, d3, and at d6. **(F):** Morphology of GCNF rescued ESCs (GCNF transfected $GCNF^{-/-}$ ESCs) was compared with wt and vector control ESCs (GFP transfected $GCNF^{-/-}$ ESCs) in undifferentiated (d0) and differentiated (d3 treated with RA) states. Scale bar = 50 μ m. Abbreviations: GCNF, germ cell nuclear factor; GFP, green fluorescent protein; RA, retinoic acid.

levels in wt ESCs was consistent with its low expression in E3.5 wt embryos [3].

GCNF Directly Inhibited Mir302 by Binding to the Promoter of Mir302

To determine whether the *Mir302a* gene is a direct target gene of GCNF in mouse ESCs, we searched for putative DR0 (AGGTCAAGGTCA: consensus) sites within the *Mir302a* promoter region. We found a predicted DR0 sequence of TGGCCTTGAAC (AGTTC AAGGCCA: lower strand) located 1,776 bp upstream of the *Mir302a* transcriptional start site (Supporting Information Fig. S2). To validate this prediction, we used both EMSAs and ChIP assays to detect whether GCNF binds to this DR0 site. EMSA results show that GCNF directly binds to the DR0 element in the *Mir302a* promoter (Fig. 4A). GCNF binding to the DR0 is undetectable in undifferentiated (day 0) ESCs, where GCNF is not expressed. The strongest

GCNF binding appears at day 1.5 then decreases as differentiation proceeds (Fig. 4A). As expected, no binding was detected in either undifferentiated or differentiated $GCNF^{-/-}$ ESCs (Fig. 4A). The migration of the binding complex could be retarded when the anti-GCNF antibody was added to the wt ESC nuclear extracts and no shifted bands were observed with the $GCNF^{-/-}$ ESC extracts. This DNA binding pattern is coincident with the GCNF expression pattern. In addition, GCNF ChIP analysis using amplicon primers that spanned DR0 element in the endogenous *Mir302a* promoter showed similar results to the EMSA analysis (Fig. 4B). The results demonstrated that *Mir302a* was a GCNF target gene in vivo during ESC differentiation.

To resolve whether GCNF regulates *Mir302a* expression, we constructed luciferase reporter plasmids driven by the *Mir302a* promoter with or without the DR0 response element. The plasmids were transfected into wt and $GCNF^{-/-}$

ESCs, and cells were induced to differentiate with RA for 1.5 days. Luciferase activity was lower in wt ESCs transfected with the reporter housing the DR0 response element than luciferase activity in *GCNF*^{-/-} ESCs (Fig. 4C). This effect is lost when a shorter promoter reporter plasmid lacking the DR0 response element was transfected into the two cell types. These results demonstrate that endogenous GCNF can directly inhibit *Mir302a* gene expression through the identified DR0 response element.

Inhibition of Mir302a Rescued Cyclin D1 Expression During *GCNF*^{-/-} ESC Differentiation

Based on the this evidence, we hypothesized that in *GCNF*^{-/-} ESCs loss of GCNF-dependent repression of the *Mir302a* gene expression leads to high levels of *Mir302a*, which in turn can inhibit cyclin D1 expression at a critical time point during early differentiation when cyclin D1 should be expressed to initiate a somatic cell cycle with a G1/S check point. To test this, we constructed two cyclin D1 3'UTR luciferase reporter plasmids. One containing the predicted endogenous cyclin D1 3'UTR *Mir302a* binding site and the second, serving as a negative control, contains the *Mir302a* binding site with mutations predicted to destroy *Mir302a* binding (Fig. 5A, Supporting Information Fig. S3). *Mir302a* and its negative control oligonucleotides as well as cyclin D1 3'UTR and its mutant luciferase reporter plasmids were cotransfected into P19 and 293 cells, respectively. Decreased luciferase activity was detected with the cotransfection of *Mir302a* and cyclin D1 3'UTR reporter compared to the activity with cotransfection of *Mir302a* negative control oligonucleotides and cyclin D1 3'UTR reporter ($p < .05$). Cotransfection of *Mir302a* and the mutant cyclin D1 3'UTR reporter plasmid did not display a significant decrease in luciferase activity compared to the *Mir302a* negative control ($p > .05$) in P19 cells or in 293 cells (Fig. 5B, 5C). These results suggest that *Mir302a* can regulate cyclin D1 expression by a posttranscriptional regulatory mechanism.

The above evidence implies that *Mir302a* can inhibit cyclin D1 expression, but we do not know if *Mir302a* can block the activation of cyclin D1 expression during ESC differentiation. To test this, we transfected *Mir302a* inhibitor oligonucleotides and negative control (NC) oligonucleotides into *GCNF*^{-/-} ESCs daily for 3 days while treating cells with RA to induce differentiation. Western blot results showed that cyclin D1 expression was reactivated in *GCNF*^{-/-} ESCs after the transfection of the *Mir302a* inhibitor (mir302i) and no cyclin D1 expression was detected in cells transfected with negative control (Mir302-NC) or mock transfection controls after 3 days of RA-induced differentiation (Fig. 5D). The data established that the high levels of *Mir302a* caused the loss of expression of cyclin D1 in *GCNF*^{-/-} ESCs.

Reintroduction of GCNF into *GCNF*^{-/-} ESCs Rescues *Mir302a* Repression and Cyclin D1 Activation

Based on the GCNF-dependent inhibition of *Mir302a* expression and the failure to activate cyclin D1 expression during *GCNF*^{-/-} ESC differentiation, we tested whether reintroduction of recombinant GCNF into *GCNF*^{-/-} ESCs could inhibit *Mir302a* over-expression and restore the activation of cyclin D1 expression. A GCNF expression (tGCNF) plasmid and a control GFP expression (tGFP) plasmid were constructed (Support-

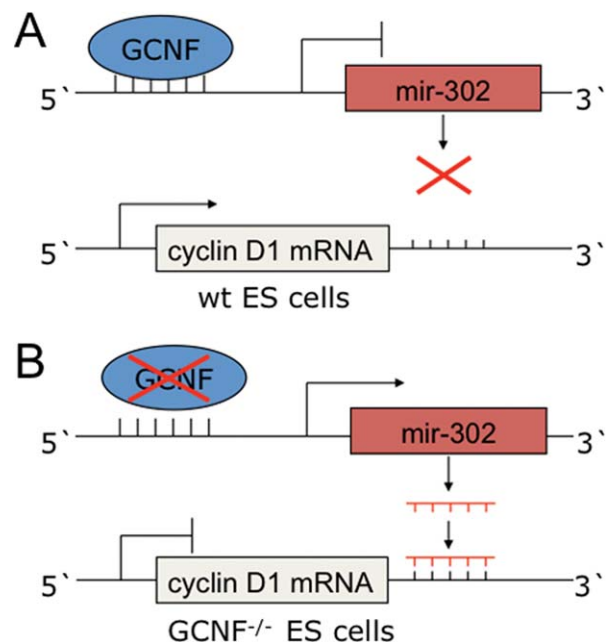


Figure 7. Molecular model of indirect activation of cyclin D1 by GCNF via *Mir302a*. **(A):** In wt ESCs, GCNF is expressed and *Mir302a* expression is inhibited, which derepresses the cyclin D1 expression. **(B):** In *GCNF*^{-/-} ESCs, GCNF is not expressed and *Mir302a* expression was derepressed, which in turn represses cyclin D1 expression. Abbreviations: ESC, embryonic stem cell; GCNF, germ cell nuclear factor.

ing Information Fig. S4A, S4B) and transfected into *GCNF*^{-/-} ESCs that express a truncated but nonfunctional GCNF lacking the DNA-binding domain. After puromycin selection, GCNF protein was detectable by Western blot in undifferentiated and differentiated FlagGCNF transfected *GCNF*^{-/-} ESCs (Fig. 6A). In addition, the repression of Oct4 was rescued after differentiation of *GCNF*^{-/-} ESCs, similar to Oct4 repression in wt ESCs at both the protein and RNA levels (Fig. 6A, 6B) because GCNF is a known repressor of Oct4 [23, 31].

The repressor of cyclin D1, *Mir302a*, was completely repressed in both undifferentiated and differentiated *GCNF*^{-/-} ESCs and was not repressed in GFP control cells (Fig. 6C). As expected, cyclin D1 expression was restored during differentiation by GCNF overexpression in *GCNF*^{-/-} ESCs. In contrast, no cyclin D1 expression was detected in the GFP control cells (Fig. 6D, 6E). The rescued expression levels of cyclin D1 during differentiation were lower than that observed in wt ESCs during differentiation. These data showed that GCNF could rescue the activation of cyclin D1 expression in *GCNF*^{-/-} ESCs.

The morphology of the FlagGCNF expressing *GCNF*^{-/-} ESCs was similar to that of differentiated wt ESCs after 3 days of differentiation, compared to the undifferentiated cell morphology in control cells (Fig. 6F). This is a demonstration of a gain-of-function experiment that demonstrates that GCNF is a repressor of Oct4 and a facilitator of ESC differentiation.

DISCUSSION

The mechanisms responsible for regulation of cyclin D1 expression during early ESC differentiation are understood

in part. The results reported here demonstrate that GCNF is a key factor regulating the activation of cyclin D1 during pluripotent stem cell differentiation. We observed that GCNF inhibited Mir302a expression, which in turn is a repressor of cyclin D1 expression through binding to its 3'UTR. GCNF-dependent repression of mir302, during ESC differentiation facilitates activation of the cyclin D1 expression (Fig. 7A). The absence of *GCNF* leads to a defective expression of cyclin D1 due to derepression of Mir302a (Fig. 7B).

In hESCs, mir-302 expression is dependent on Oct4 expression. Undifferentiated hESCs express mir302 at higher levels than differentiated hESCs [3]; in mouse embryos, although Oct4 was expressed at higher levels at E3.5 than embryos at E7.5, the expression of mir-302 at E3.5 was lower than embryos at E7.5 [3]. There are possibly different regulatory mechanisms between human and mouse that control mir302 expression. There are no publications describing whether GCNF plays the same role in hESCs in regulation of cyclin D1 via direct repression of miR-302.

Loss of GCNF expression influences both ESC differentiation and cell cycle. Expression of the endoderm marker AFP was retarded in GCNF KO ESCs. The mesoderm marker Brachyury was expressed at extremely high levels, this observation needs to be further investigated since it was recently shown that Oct4 plays a role in mesoderm differentiation [32]. In undifferentiated ESCs, inactivation of GCNF increased the number of cells in S phase, which is probably responsible for the faster proliferation. Thus, the higher proliferation of differentiated *GCNF*^{-/-} ESCs was probably related to the continued expression of pluripotent genes, such as Oct4, and so forth.

Cyclin D1 plays an important role in the gene regulatory processes during embryonic development [33–35]. Cyclin D1 function cannot be genetically rescued by cyclin D2 [36] and embryogenesis proceeds normally in cyclin D2 and cyclin D3-deficient mice, revealing a unique requirement for cyclin D1 in vivo within selected tissues. Cyclin D1 has also been shown to interact with tissue-specific transcription factor thyroid hormone receptor beta, androgen receptor, and estrogen receptor alpha [33, 37–39]. Re-expression of GCNF in *GCNF*^{-/-} ESCs not only restored the repression of Mir302a but also restored the activation of cyclin D1 expression. Importantly, D

type cyclins are indispensable for the progression of adult mammalian proliferating cells through the G1 phase of the cell cycle with each cell type displaying a specific type of D-cyclin expression and dependency [40].

These findings are in line with a recent report describing the capacity of Mir302a to bind the cyclin D1 3'UTR, contributing to its repression of cyclin D1 expression [3]. Loss of GCNF function results in derepression of Mir302a in *GCNF*^{-/-} ESCs leading high levels compared to that in wt ESCs. Interestingly, Mir302a was found to be a direct target gene of GCNF that inhibits Mir302a expression by binding to a DR0 response element located in its promoter [19, 24, 41].

CONCLUSION

Activation of cyclin D1 expression proceeds in a narrow window during ESC differentiation. GCNF inhibits Mir302a expression, leading to the activation of cyclin D1 in wt ESCs. *Mir302a* expression is activated in *GCNF*^{-/-} ESCs, which inhibits cyclin D1 via Mir302a binding to the cyclin D1 mRNA 3'UTR. These findings unveil a mechanistic connection between GCNF and cyclin D1, and thereby contribute to our understanding of the molecular basis of the cyclin D1 activation during early embryonic development and the ESC differentiation.

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AUTHOR CONTRIBUTIONS

H.W.: conception and design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing; X.W.: collection and/or assembly of data and data analysis and interpretation; T.A.: collection and/or assembly of data; T.Z.: data analysis and interpretation; A.J.C.: conception and design, data analysis and interpretation, manuscript writing, financial support, and final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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