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GCNF-dependent activation of cyclin D1 expression via repression of Mir302a during ES cell differentiation

Hongran Wang[1], **Xiaohong Wang**[1,2], **Trevor K. Archer**[3], **Thomas P. Zwaka**[4], and **Austin J. Cooney**[1,*]

[1]Departments of Molecular and Cellular Biology and Surgery, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030

[2]Stem cell center, Texas Heart Institute, 6770 Bertner Avenue, Houston, Texas 77030

[3]Chromatin and Gene Expression Section, Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

[4]Black Family Stem Cell Institute, Department of Developmental & Regenerative Medicine, Icahn School of Medicine at Mount Sinai, 1425 Madison Ave, New York, NY 10029

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 (ES) cell differentiation as a model of early development. During our study of *GCNF* knock out ($GCNF^{-/-}$) ES cells, we discovered that loss of GCNF leads to the repression of cyclin D1 activation during ES cell differentiation. This was determined to be an indirect effect of de-regulation 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 $GCNF^{-/-}$ ES cells restored cyclin D1 expression. Similarly over-expression of GCNF during differentiation of $GCNF^{-/-}$ ES cells 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.

Keywords

Cyclin D1; GCNF; microRNA; Mir302; ES cells

*To whom correspondence should be addressed Tel: +1-713-798-6250, Fax: +1-713-790-1275, acooney@bcm.edu.

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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 (ES) cells [2, 3]. Cyclin D1 regulates the cell cycle and proliferation through interaction with CCNDBP1, Retinoblastoma protein, Cyclin dependent kinase (CDK)-4, CDK-6 and PCNA [4–7]. Cyclin D1 was found not only in proliferating cells (e.g. kidney, intestine) but also in the non-dividing 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, hypolastic mammary glands and developmental neurological abnormalities [10, 11]. Mouse ES cells 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 check point. ES cells employ a D-cyclin-independent cell cycle machinery to maintain both proliferation and inhibition of differentiation in response to phoshphatidyl inositol-3PI3 kinase (PI3K) signaling in undifferentiated ES cells [12–14]. Early embryonic pluripotent cells and derivative ES cells 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 ES cells to cyclin D-dependent cell cycle as ES cells start to differentiate. A crucial question is what mechanism underlies this switch and what factors regulate it during ES cell 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 ES cells [16]. Mir302a was reported to inhibit cyclin D1 expression by binding the cyclin D1 3` UTR in human ES cells [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 ES cell 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*$^{-/-}$ ES cells 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 ES cells 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 mES cell 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*$^{-/-}$ ES cells 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 ES cell differentiation.

MATERIALS AND METHODS

ES cell lines and differentiation and embryos

Wild type and *GCNF*$^{-/-}$ ES cells 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. ES cells were Maintained in DMEM media (Life) with 15% FBS (Hyclone), 1 × MEM nonessential amino acids (Life), 2 mM L-glutamine (Life), 0.1 mM 2-mercaptoethanol (Sigma), 1000 U/ml LIF (Thermo Scientific). ES cells were induced to differentiate by treatment with 1 μM RA (Sigma) 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, 1×10^6 wt and *GCNF*$^{-/-}$ ES cells were injected into the rear leg muscle of 4 to 6 week-old severe combined immuno-deficient (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 de-paraffinized using xylene and ethanol, and then rehydrated in water for 1 minute. The sections were blocked with 10% horse serum and 1% BSA. Primary antibodies against cyclin D1 (Thermo scientific) were diluted 1:200 and normal rabbit IgG (Santa Cruz) was used as control. Secondary antibody Texas-red labeled goat anti-rabbit antibodies (Vector Laboratories) were diluted 1:500.

Analysis of cell cycle by flow cytometry

wt and GCNF$^{-/-}$ cells were trypsinized, washed twice with PBS, re-suspended 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), 100 μg/ml DNase-free RNase (Sigma) was added to each sample (2×10^6 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) 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). RNA samples were reverse transcribed to cDNA using SuperScript III First-Strand Synthesis System

(Invitrogen). PCR primers used in this paper were listed as supplemental information table S1. Mir302a was analyzed with hsa-miR302 TaqMan® MicroRNA Assay, and U6 snRNA as control using Taqman reagents (Invitrogen). Northern blot was also used to analyzed the Mir302 (probe: 5`TCACCAAAACATGGAAGCACTTA 3`) expression of wt and *GCNF*$^{-/-}$ ES cells. Briefly, 30 mg of total RNA was resolved in a 15% denaturing polyacrylamide gel containing 7 M urea in 0.5XTBE buffer system and transferred onto Zeta-Probe membrane (BioRad) in 0.5XTBE. Mir302 probe oligonucleotides were radioactively labeled with ^{32}P-ATP (MP Biomedicals) and T4 kinase (Invitrogen). Ultraviolet light-cross linked membranes were hybridized with radioactively-labeled DNA probe at 45°C in Quickhyb solution (Stratagene) and washed with 2xSSC, 0.1% 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) and GCNF were used at a 1:1000 dilution; cyclin D1 antibodies (Thermo scientific) were used at a1:1000 dilution. β-actin antibodies (Sigma) were used at a 1:5000 dilution. All antibodies was diluted in TBS with 5% fat free dry milk. The secondary antibodies, goat anti-mouse IgG-HRP (Santa Cruz) and goat anti-rabbit IgG-HRP (Santa Cruz) were used at a 1:2000 dilution. HRP activity was detected using chemiluminescence with Pierce ECL Western Blotting Substrate kit (Thermo scientific). The emitted light was detected by photographic film.

Chromatin immunoprecipitation analysis

Sonicated DNA samples of undifferentiated and differentiated ES cells 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) with an ABI 7900HT real-time PCR machine. The following primer sets spanning the Mir302a promoter were used: Mir302a forward: 5'CTGGCTGTCCTGGAACTCACT3', reverse: 5'TCATGCCTTTGATCCCAGCACT3'.

Electrophoretic mobility shift assays (EMSAs)

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_ protease inhibitor cocktail [Roche]). Nuclear extracts were prepared from undifferentiated and differentiated wt and GCNF$^{-/-}$ ES cells at different time points of RA treatment, 1, 2, 3, 4, 5 and 6 days. Oligonucleotides that contained complementary sequences (Forward: 5`GTAGACCAGGCTGGCCTTGAACTCAGAAAT3`; Reverse: 5`CGGATTTCTGAGTTCAAGGCCAGCCTGGT3`) 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. EMSAs were performed as described by Garner and Revzin [26].

Luciferase reporter assays

pMIR-report luciferase plasmid (Ambion, Austin, TX) 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 was also cloned as a control (Figure 5A, supplementary information, Figure 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 ES cell DNA. The primer sets was listed below:

Forward1: 5`TCGGTACCAAAGAATAAAGCCTTA3`;

Forward2: 5` TCGGTACCTCTGGAAGAGCAGTCA3`;

Reverse: 5`CCCTCGAGTCCTGTTCTGTCCTCG3`; 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 ul of passive lysis buffer (Promega) 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) and miRIDIAN microRNA Inhibitor Negative Control #1 (Mir302NC) (Dharmacon) were transfected into 7.5×10^5 *GCNF*$^{-/-}$ ES cells in each well of 6 well plates, respectively, using Lipofectamine 2000 (Invitrogen) reagent according to the manufacturer's instructions. After transfection, the cells were treated with RA to induce ES cell 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 ES cell mRNA. The resulting cDNA was cloned into the Gateway/Topo TA vector (Invitrogen), and was then recombined into the destination retroviral plasmid with the mscv promoter by Gateway LR Clonase (Invitrogen). For packaging the virus, 1.5×10^6 BOSC cells were plated in 6 well of plates (10% FBS DMEM), and were cultured overnight. Mscv-GCNF, mscv-GFP with packaging plasmid pDuo were introduced into each 6 cm plate with BOSC cells [27] respectively using Fugene 6 transfection reagent (Roche) 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 hr of infection, the media was replaced with 2.5 ml fresh ES cell medium. The transfected

cells were selected for two weeks with 1 μg/ml of puromycin after three 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 ± standard deviation (SD). Student's T-test was performed to determine the differences among grouped data. * indicates statistically significance with $P<0.05$.

RESULTS

Loss of GCNF alters gene expression and proliferation in ES cells

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 ES cell colonies. The morphology of the *GCNF* knockout (*GCNF*$^{-/-}$) ES cell colonies compared to wild type (wt) ES cells lacks multiple layers, typically characteristic of mouse ES cell colonies, rather they form a single layer on gelatin-coated plates; appearing like human ES cells in nature (Figure 1A). After the ES cells were induced to differentiate with RA, the *GCNF*$^{-/-}$ ES cells still maintained an undifferentiated morphology: round and small, compact nucleus, with a high nucleus-to-cytoplasm volume ratio, even at day 6 of differentiation. In contrast, the morphology of RA treated wt ES cells possesses characteristics of a differentiated cell, they are larger and irregular in shape and they have a lower nucleus-to-cytoplasm volume ratio (Figure 1A).

GCNF$^{-/-}$ ES cells also showed a faster proliferation rate than wt ES cells in both undifferentiated and differentiated states *in vitro* (Figure 1B). *In-vivo* proliferation of both ES cell types was also evaluated by injection of undifferentiated and differentiated (d3 and d6) wt or *GCNF*$^{-/-}$ ES cells into the SCID mice, respectively. Four weeks later, the size of the teratomas from *GCNF*$^{-/-}$ ES cells was found to be significantly larger than those derived from wt ES cells in all three corresponding samples (Figure 1C). In general, teratoma formation in undifferentiated ES cells was the largest, and teratomas from day 6 differentiated ES cells were the smallest. The size of teratomas from *GCNF*$^{-/-}$ ES cells was consistently larger relative to those derived from wt cells (Figure 1C) correlating with the maintenance of *Oct4* gene expression in differentiated GCNF$^{-/-}$ ES cells and the down regulation of Oct4 in wt ES cells (Figure 1D). Maintenance of an undifferentiated morphology is most probably caused by the expression of relatively high levels of pluripotency genes in differentiated *GCNF*$^{-/-}$ ES cells [23]. In addition, inactivation of GCNF did not affect the overall ability to form embryoid bodies (EBs) (Supplementary information, Figure S1).

The expression pattern of lineage marker genes was altered between wt EBs and *GCNF*$^{-/-}$ EBs. *Afp*, a marker of endoderm (Figure 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 (Figure 1F). *Sox1*, a marker of neurectoderm, 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 (Figure 1G). Thus, loss of GCNF has changes the typical characteristics of ES cells, including more rapid proliferation, which prompted us to analyze expression of cell cycle regulatory factors.

Failure to activate cyclin D1 expression during GCNF$^{-/-}$ ES cell 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] and in undifferentiated mouse ES cells 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 ES cell system that we derived from blastocyst outgrowths. Wt and GCNF$^{-/-}$ ES cells were induced to differentiate with RA treatment after LIF removal. RNA and protein were isolated from undifferentiated ES cells (at day 0) (d0) and differentiated ES cells 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 ES cells and quickly increased after differentiation in wt ES cells (Figure 2A). Western blot of whole cell lysates using anti-GCNF, Oct4 and cyclin D1 antibody showed that Oct4 expression is maintained in GCNF$^{-/-}$ ES cells during differentiation compared to the rapid decrease in wt ES cells during differentiation (Figure 2B) [22]; cyclin D1 was expressed at low levels in undifferentiated ES cells and was quickly activated after RA induced differentiation in wt ES cells (Figure 2B). In contrast cyclin D1 expression decreased rather than increased at both the mRNA and protein levels after differentiation of *GCNF*$^{-/-}$ ES cells. Although cyclin D1 was expressed in undifferentiated *GCNF*$^{-/-}$ ES cells, cyclin D1 mRNA and protein levels are lower than that of wt ES cells and was not induced upon ES cell differentiation with RA treatment (Figure 2A, 2B).

To further investigate the dynamic change of cyclin D1 expression during ES cell differentiation, we induced *GCNF*$^{-/-}$ ES cells to differentiate by EB formation without RA treatment. Western blot of whole cell lysates demonstrated that Oct4 expression is maintained during GCNF$^{-/-}$ EB formation. Surprisingly, GCNF was still transiently induced in wt ES cells during EB formation, similar to RA induced differentiation (Figure 2C); cyclin D1 was not activated till day 12 of differentiation in *GCNF*$^{-/-}$ EBs, while cyclin D1 expression was activated in wt differentiated EBs (Figure 2C). These results suggested that GCNF plays a crucial role in regulating the induction of cyclin D1 expression during ES cell differentiation.

To determine if these *in vitro* results in ES cells 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 ES cell 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 proliferative. In contrast, no cyclin D1 protein expression was detected in E8.5 *GCNF*$^{-/-}$ embryos (Figure 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. By using fluorescence-activated cell sorting (FACS) analysis to evaluate the cell cycle profiles of propidium iodide stained cells we were able to determine differences between wt and GCNF$^{-/-}$ ES cells (Figure 2E), The cell number of G1 phase of undifferentiated GCNF$^{-/-}$ cells was lower than wt undifferentiated ES and increased during cell differentiation, more than observed in wt ES cells at day 6 (Figure 2E). In contrast, the number of S phase GCNF$^{-/-}$ ES cells was higher than WT ES cells in undifferentiated state, and then decreased to a lower level than that of WT ES cells after differentiation at day 6 (Figure 2E). G2+M phase cells decreased both in wt and in GCNF$^{-/-}$ ES cells (Figure 2E). These results indicate that GCNF regulates the cell cycle during ES cell differentiation.

Mir302a expression dramatically increased in GCNF$^{-/-}$ ES cells

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 ES cell 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 ES cell differentiation demonstrated that Mir302 was highly up-regulated in *GCNF*$^{-/-}$ ES cells relative to wt ES cells [30]. To validate if Mir302a was highly expressed in *GCNF*$^{-/-}$ ES cells, qRT-PCR was used to detect Mir302a expression in wt and *GCNF*$^{-/-}$ ES cells. High level expression of Mir302a was found in undifferentiated and early differentiating *GCNF*$^{-/-}$ ES cells (Figure 3A) compared to the low levels of Mir302a in both undifferentiated and early differentiating wt ES cells (Figure 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*$^{-/-}$ ES cells; no Mir302 was detected in wt ES cells (Figure 3B). Mir302a expression at low levels in wt ES cells 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 ES cells, we searched for putative DR0 (AGGTCAAGGTCA: consensus) sites within the Mir302a promoter region. We found a predicted DR0 sequence of TGGCCTTGAACT (AGTTCAAGGCCA: lower strand) located 1,776 bp upstream of the Mir302a transcriptional start site (TSS) (Supplementary information, Figure S2). To validate this prediction, we used both electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (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 (Figure 4A). GCNF binding to the DR0 is undetectable in undifferentiated (day 0) ES cells, where GCNF is not expressed. The strongest GCNF binding appears at day 1.5 then decreases as differentiation proceeds (Figure 4A). As expected, no binding was detected in either undifferentiated or differentiated *GCNF*$^{-/-}$ ES cells (Figure 4A). The migration of the binding complex could be retarded when the anti-GCNF antibody was added to the wt ES cell nuclear extracts and no shifted bands were observed with the *GCNF*$^{-/-}$ ES cell 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 (Figure 4B). The results demonstrated that *Mir302a* was a GCNF target gene *in-vivo* during ES cell 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*$^{-/-}$ ES cells, and cells were induced to differentiate with RA for 1.5 days. Luciferase activity was lower in wt ES cells transfected with the reporter housing the DR0 response element than luciferase activity in *GCNF*$^{-/-}$ ES cells (Figure 4C). This affect 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$^{-/-}$ ES cell differentiation

Based on the this evidence we hypothesized that in *GCNF*$^{-/-}$ ES cells 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 (Figure 5A, supplementary Figure S3). Mir302a and its negative control oligonucleotides as well as cyclin D1 3`UTR and its mutant luciferase reporter plasmids were co-transfected into P19 and 293 cells, respectively. Decreased luciferase activity was detected with the co-transfection of Mir302a and cyclin D1 3`UTR reporter compared to the activity with co-transfection of Mir302a negative control oligonucleotides and cyclin D1 3`UTR reporter ($P<0.05$). Co-transfection 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 ($P>0.05$) in P19 cells or in 293 cells (Figure 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 ES cell differentiation. To test this, we transfected Mir302a inhibitor oligonucleotides and negative control (NC) oligonucleotides into *GCNF*$^{-/-}$ ES cells daily for three days while treating

cells with RA to induce differentiation. Western blot results showed that cyclin D1 expression was reactivated in *GCNF*$^{-/-}$ ES cells 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 (Figure 5D). The data established that the high levels of Mir302a caused the loss of expression of cyclin D1 in *GCNF*$^{-/-}$ ES cells.

Re-introduction of GCNF into GCNF$^{-/-}$ ES cells 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*$^{-/-}$ ES cell differentiation, we tested whether re-introduction of recombinant *GCNF* into *GCNF*$^{-/-}$ ES cells 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 (Supplementary information, Figure S4A, S4B) and transfected into *GCNF*$^{-/-}$ ES cells that express a truncated but non-functional GCNF lacking the DNA-binding domain. After puromycin selection, GCNF protein was detectable by western blot in undifferentiated and differentiated FlagGCNF transfected *GCNF*$^{-/-}$ ES cells (Figure 6A). In addition, the repression of Oct4 was rescued after differentiation of *GCNF*$^{-/-}$ ES cells, similar to Oct4 repression in wt ES cells at both the protein and RNA levels (Figure 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*$^{-/-}$ ES cells and was not repressed in GFP control cells (Figure 6C). As expected, cyclin D1 expression was restored during differentiation by GCNF overexpression in *GCNF*$^{-/-}$ ES cells. In contrast no cyclin D1 expression was detected in the GFP control cells (Figure 6D, 6E). The rescued expression levels of cyclin D1 during differentiation were lower than that observed in wt ES cells during differentiation. These data showed that GCNF could rescue the activation of cyclin D1 expression in *GCNF*$^{-/-}$ ES cells.

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

DISCUSSION

The mechanisms responsible for regulation of cyclin D1 expression during early ES cell 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 ES cell differentiation facilitates activation of the cyclin D1 expression (Figure 7A). The absence of *GCNF* leads to a defective expression of cyclin D1 due to de-repression of Mir302a (Figure 7B).

In hES cells, mir-302 expression is dependent on Oct4 expression. Undifferentiated hES cells express mir302 at higher levels than differentiated hES cells [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 ES cell differentiation and cell cycle. Expression of the endoderm marker AFP was retarded in GCNF KO ES cells. 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 ES cells, 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$^{-/-}$ ES cells was probably related to the continued expression of pluripotent genes, such as Oct4, etc.

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$^{-/-}$ ES cells 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 de-repression of Mir302a in *GCNF*$^{-/-}$ ES cells leading high levels compared to that in wt ES cells. 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 ES cell differentiation. GCNF inhibits Mir302a expression, leading to the activation of cyclin D1 in wt ES cells. *Mir302a* expression is activated in *GCNF*$^{-/-}$ ES cells, which inhibits cyclin D1 via Mir302a binding to the cyclin D1 mRNA 3`UTR. Our current 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 ES cell differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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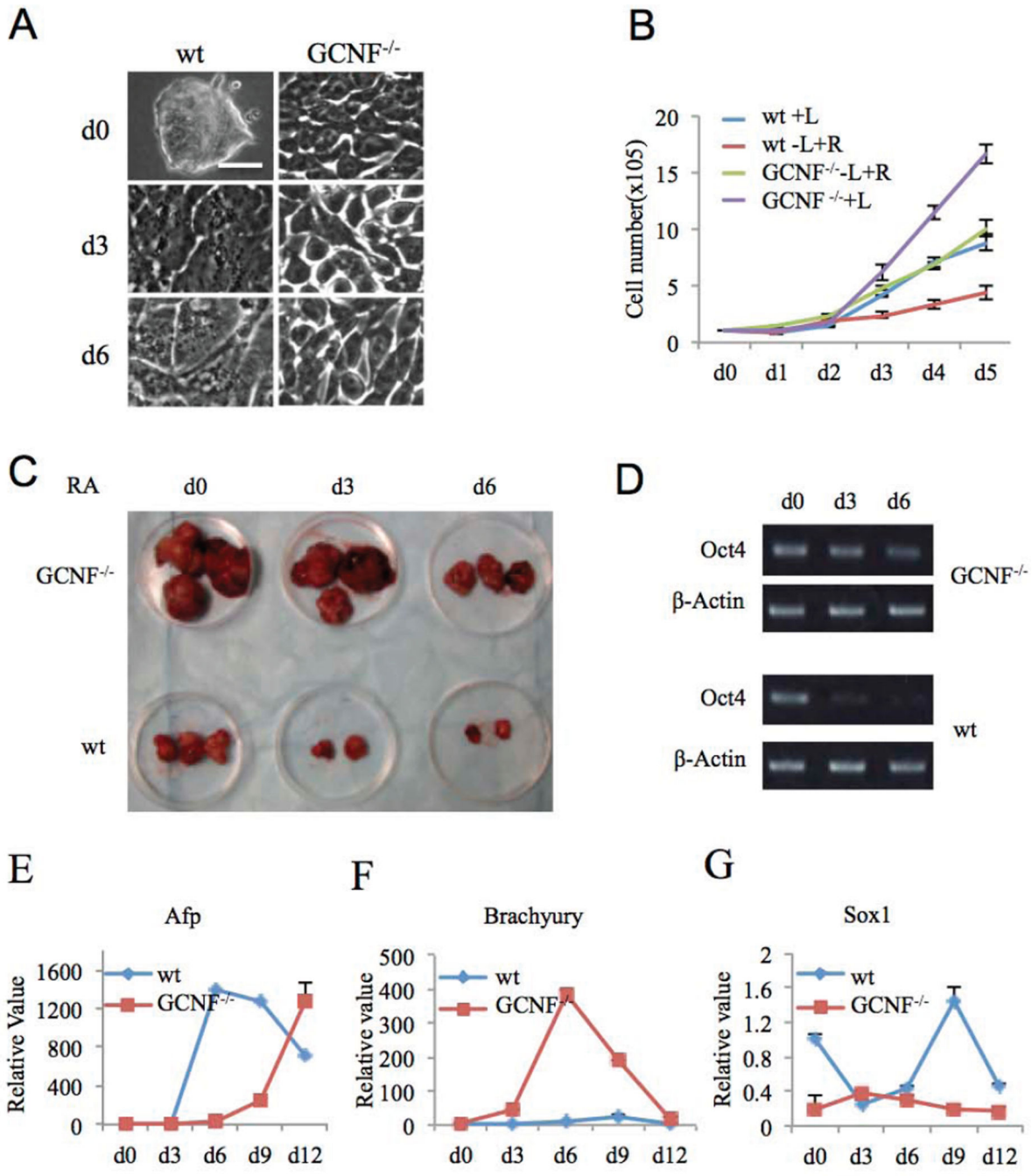

Figure 1.

GCNF promotes cell proliferation and loss of GCNF expression alters ES cell colony morphology. (A) Wt and GCNF$^{-/-}$ ES cells were plated on gelatin coated dishes, the undifferentiated ES celsl wwre cultured in ES cell media in order to maintain the undifferentiated state; the differentiated ES cells were induced by RA and LIF removal. The morphology of wt and *GCNF*$^{-/-}$ ES cells was observed under microscopy in undifferentiated (d0) state and RA induced differentiated state at d3 and d6. Scale bar: 20 μm. (B) Cells were plated on gelatin-coated plates and were counted daily for 5 days.

Undifferentiated cells (d0) were cultured in ES cell media and differentiated cells were cultured in the media without LIF and with RA. (C) 1×10^6 wt and *GCNF*$^{-/-}$ ES cells were injected in SCID 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*$^{-/-}$ ES cells at related different time points: d0, d3 and d6. (E) Undifferentiated wt and *GCNF*$^{-/-}$ ES cells RNAs were cultured on gelatin coated dishes in ES cell media; differentiated ES cells were cultured on Petri dishes in differentiated ES cell media and then the cells were aggregated into 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.

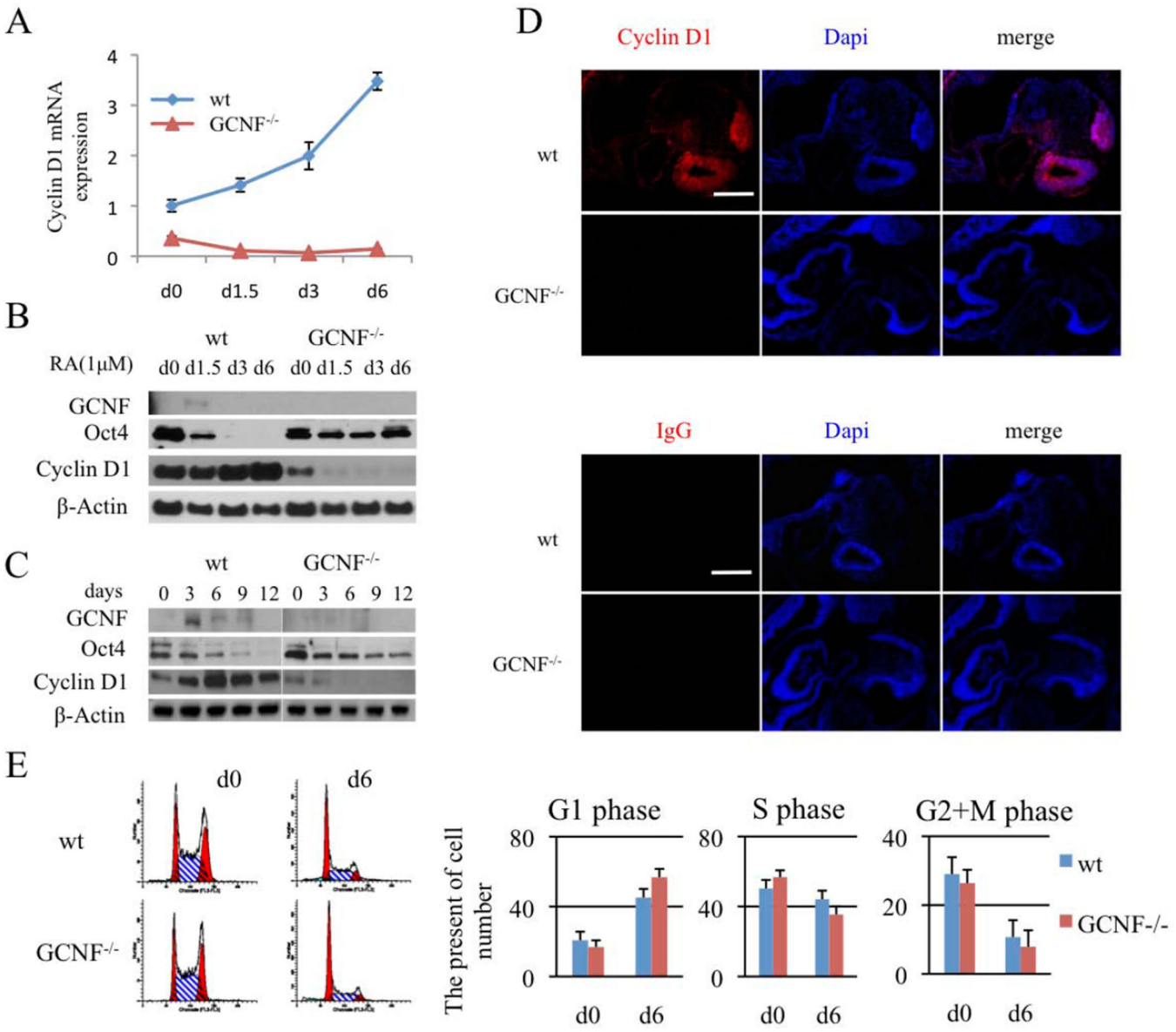

Figure 2.

Loss of *GCNF* expression fails to activate cyclin D1 expression. (A) mRNA was isolated from wt ES cells and GCNF$^{-/-}$ ES cells in undifferentiated (d0) and differentiated states (d1.5, d3, d6) induced with RA, and cyclin D1 expression was analyzed by Q-RT-PCR. Results were normalized to GAPDH expression. Error bars represent ± SD. (B) Whole cell lysate 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 EB 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 μm. (E) The cell cycle analysis of wt ES cells and GCNF$^{-/-}$ ES cells in undifferentiated and differentiated (at days 6) state by FACS.

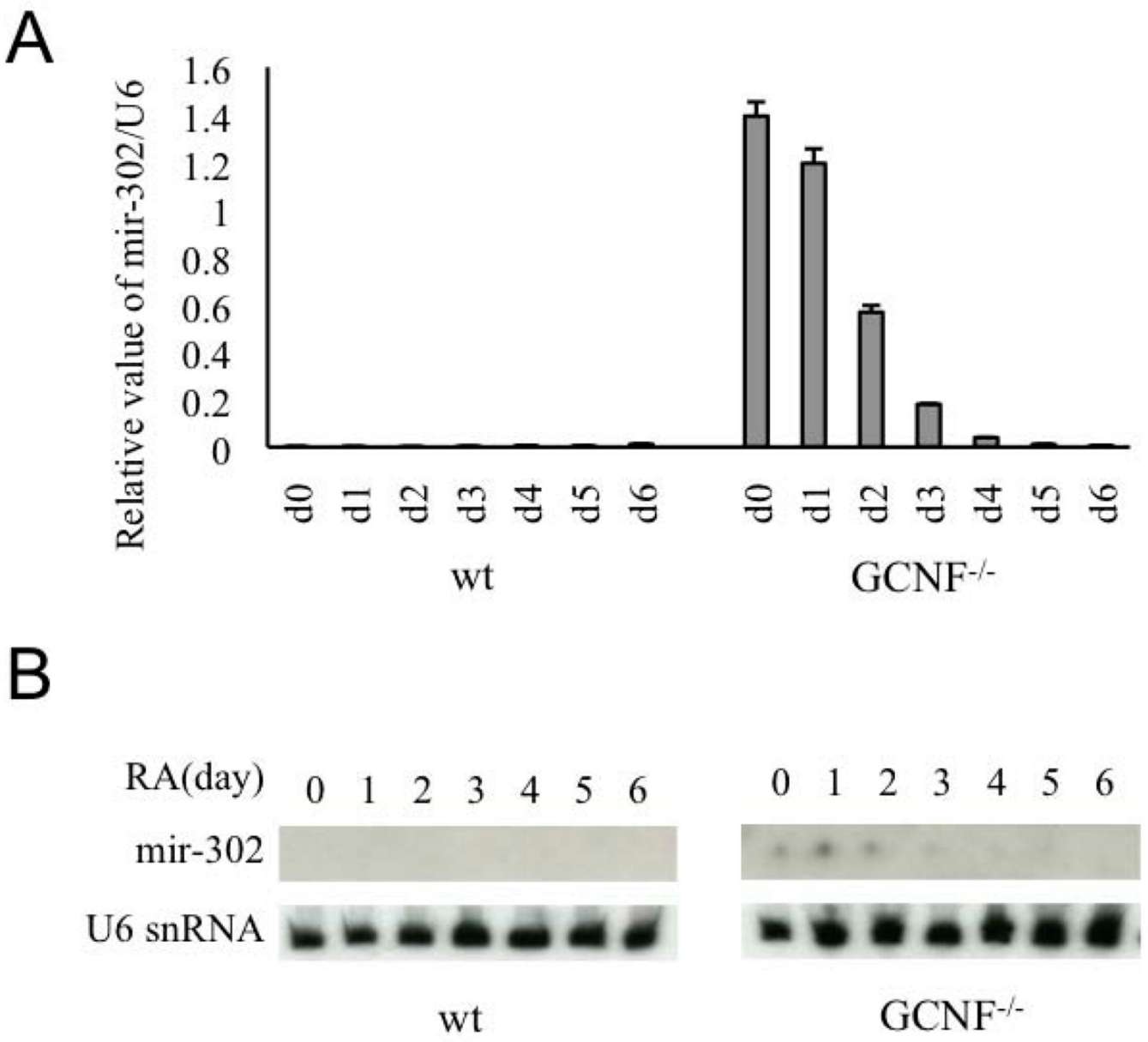

Figure 3.

Mir302a expression in wt and *GCNF*$^{-/-}$ ES cells. (A) Mir302 expression was analyzed by Q-RT-PCR in wt and GCNF$^{-/-}$ ES cells in the undifferentiated state (d0) and in a differentiated state (RA treatment for d1, d2, d3, d4, d5, d6). U6 snRNA was used as a control. (B) Mir302 expression was analyzed by northern blot in wt and *GCNF*$^{-/-}$ ES cells in the undifferentiated state (d0) and in a differentiated state (RA treatment for d1, d2, d3, d4, d5, d6). U6 snRNA was used as a loading control.

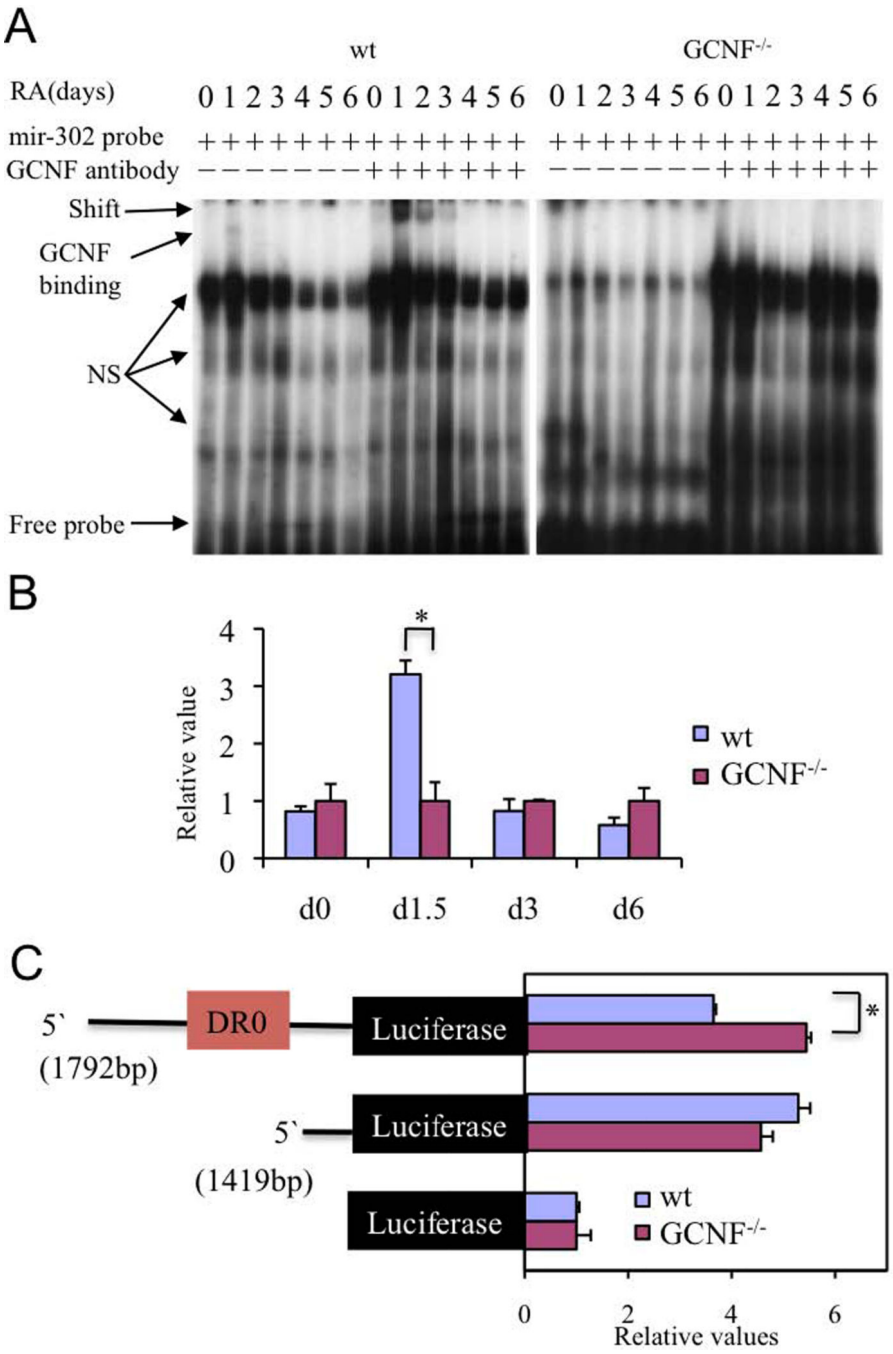

Figure 4.

GCNF directly inhibits Mir302 expression by binding to a DR0 element in the promoter of *Mir302*. (A) EMSA 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) ChIP analysis of GCNF binding to the DR0 within the Mir302 promoter was used to assess binding *in vivo* (*P<0.05). (C) Luciferase reporter assays were used to analyze the GCNF-dependent inhibition of Mir302 transcription (*P<0.05).

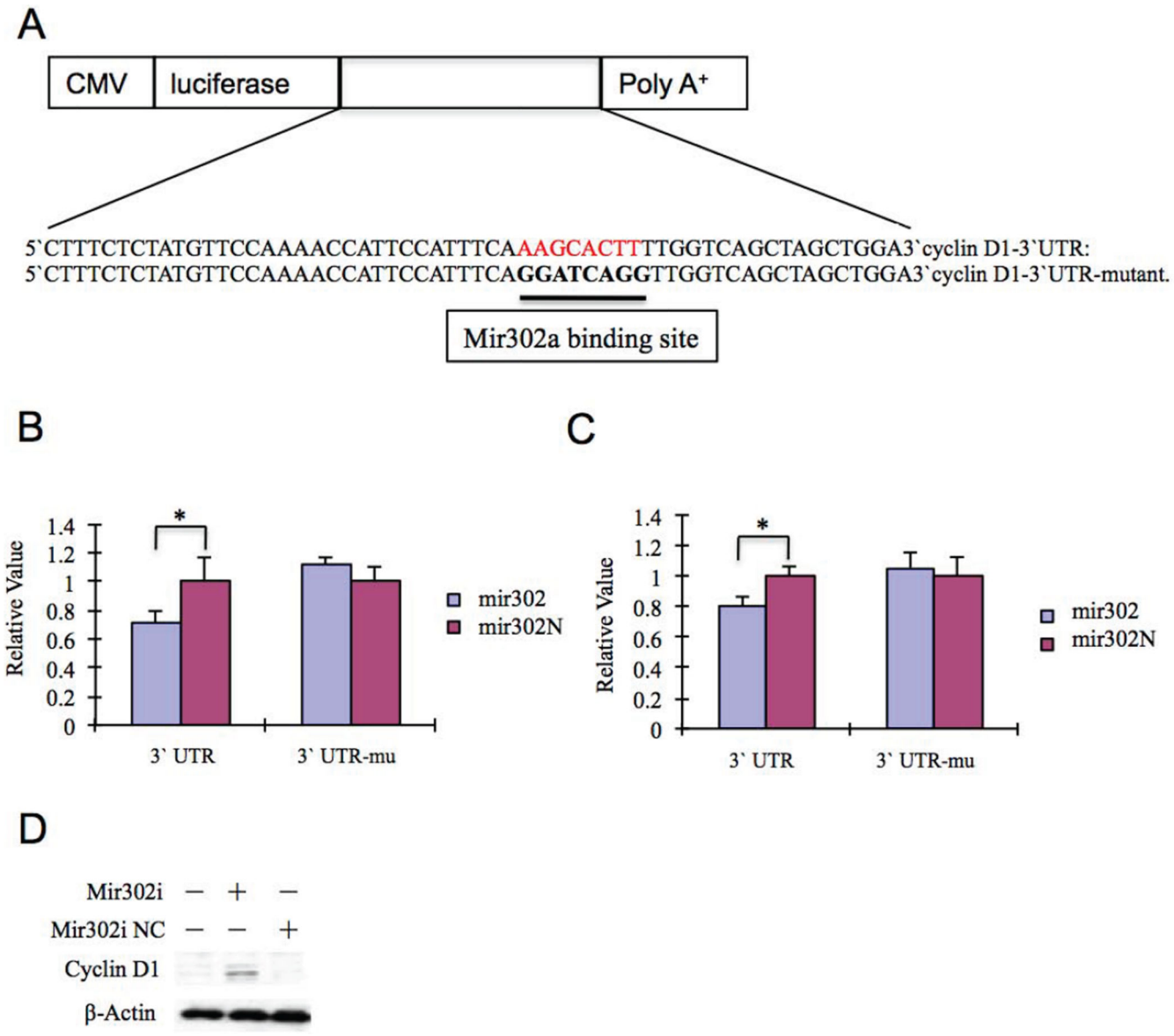

Figure 5.

Inhibition of Mir302a expression rescued cyclin D1 expression during *GCNF*$^{-/-}$ ES cell differentiation. (A) Luciferase reporter plasmids were constructed with pMiR-reporter vector into which the cyclin D1 3' ÙTR was inserted, containing the Mir302 binding site or a mutant cyclin D1 3' ÙTR as a control. (B) Relative luciferase activities were analyzed in P19 cells after a Mir302 mimic was co-transfected with the normal and mutant cyclin D1 3` UTR reporters (*P<0.05). (C) Relative luciferase activities were analyzed in 293 cells after a Mir302 mimic was co-transfected with the normal and mutant cyclin D1 3` UTR reporters (*P<0.05). (D) Mir302 inhibitor (mir302i) and a mir302 negative control (mir302 NC) were transfected into *GCNF*$^{-/-}$ ES cells, which were then induced to differentiate with RA for 3 days. Western blot of cyclin D1 expression was performed with whole cell isolates.

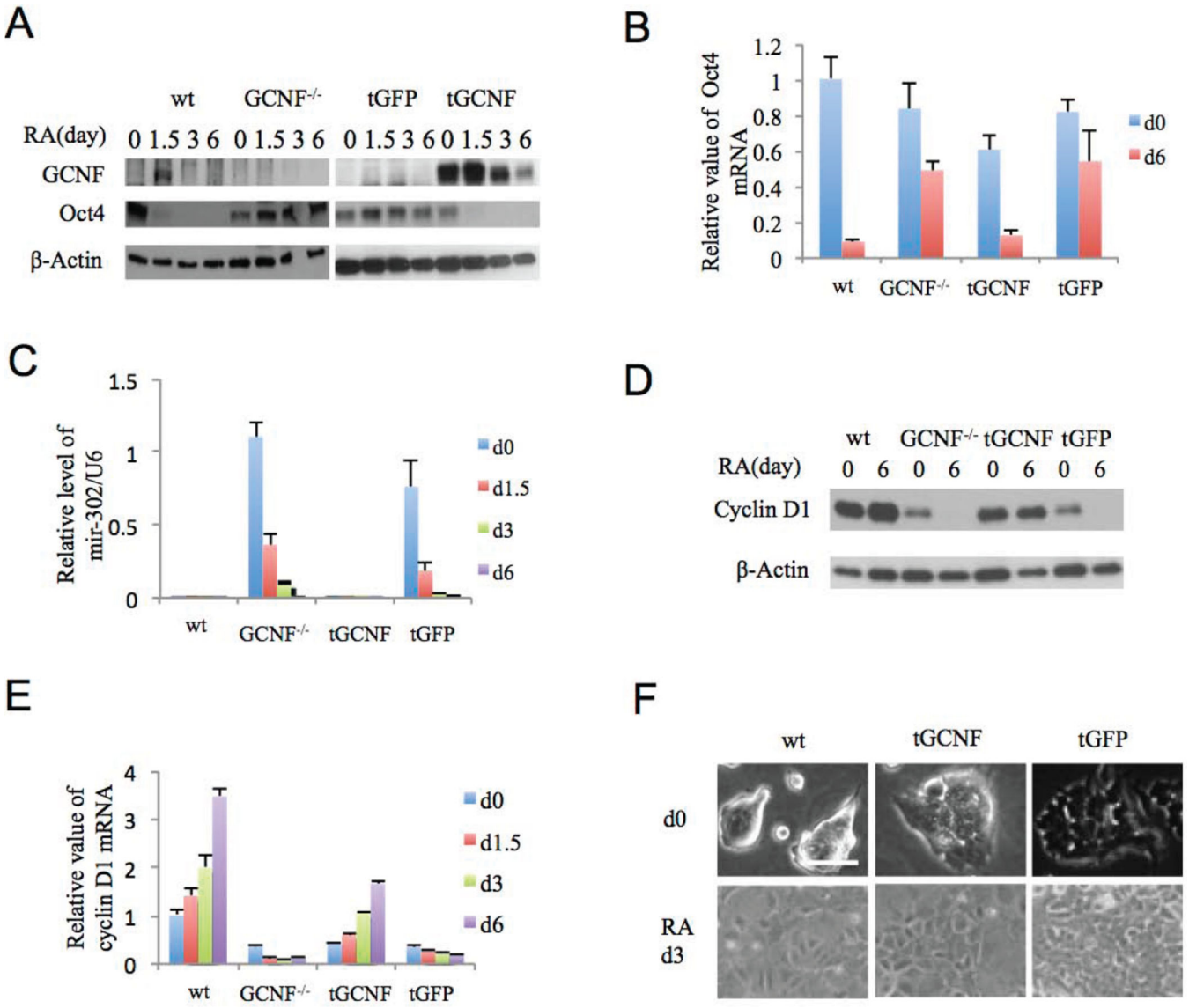

Figure 6.

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*$^{-/-}$ ES cells. Protein levels of GCNF and Oct4 were analyzed by western blot in wt ES cells, *GCNF*$^{-/-}$ ES cells, GFP expressing *GCNF*$^{-/-}$ ES cells (tGFP) and GCNF transfected *GCNF*$^{-/-}$ ES cells (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 ES cells, *GCNF*$^{-/-}$ ES cells, GCNF transfected *GCNF*$^{-/-}$ ES cells and GFP transfected *GCNF*$^{-/-}$ ES cells during differentiation. (C) Mir302 expression was analyzed in wt ES cells, *GCNF*$^{-/-}$ ES cells, GCNF transfected *GCNF*$^{-/-}$ ES cells and GFP transfected *GCNF*$^{-/-}$ ES cells by qRT-PCR during differentiation. (D) Cyclin D1 protein levels of were analyzed by western blot in wt ES cells, *GCNF*$^{-/-}$ ES cells, GCNF transfected *GCNF*$^{-/-}$ ES cells and GFP transfected *GCNF*$^{-/-}$ ES cells at d0 (undifferentiated) and at d6 (differentiated). (E) Cyclin D1 mRNA levels were analyzed by

qRT-PCR in wt ES cells, *GCNF*$^{-/-}$ ES cells, GCNF transfected *GCNF*$^{-/-}$ ES cells and GFP transfected *GCNF*$^{-/-}$ ES cells at d0, d1.3, d3 and at d6. (F) Morphology of GCNF rescued ES cells (GCNF transfected *GCNF*$^{-/-}$ ES cells) were compared with wt and vector control ES cells (GFP transfected *GCNF*$^{-/-}$ ES cells) in undifferentiated (d0) and differentiated (d3 treated with RA) states. Scale bar: 50 μm.

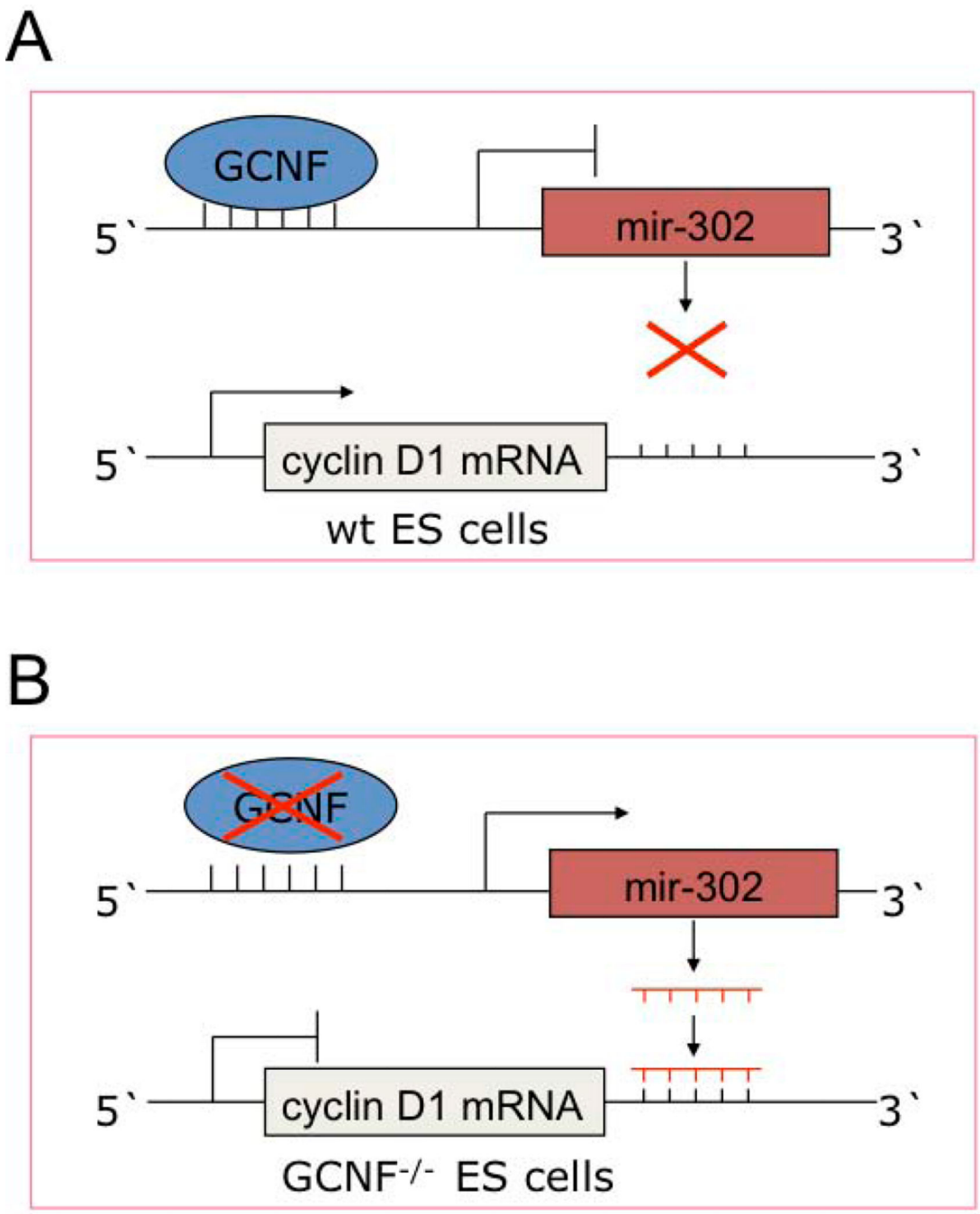

Figure 7.

Molecular model of indirect activation of cylin D1 by GCNF via Mir302a. (A) In wt ES cells, GCNF is expressed and Mir302a expression is inhibited, which de-represses the cyclin D1 expression. (B) In *GCNF*$^{-/-}$ ES cells, GCNF is not expressed and Mir302a expression was de-repressed, which in turn represses cyclin D1 expression.