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Embryonic Stem Cell Specific MicroRNAs Regulate the G1/S Transition and Promote Rapid Proliferation

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Abstract

Dgcr8 knockout embryonic stem (ES) cells lack microprocessor activity and hence all canonical microRNAs (miRNAs). These cells proliferate slowly and accumulate in G1 phase of the cell cycle1. Here, by screening a comprehensive library of individual miRNAs in the background of the *Dgcr8* knockout ES cells, we report that multiple ES cell-specific miRNAs, members of the miR-290 family, rescue the ES cell proliferation defect. Furthermore, rescued cells no longer accumulate in the G1 phase of the cell cycle. These miRNAs function by suppressing several key regulators of the G1/S transition. These results show that post-transcriptional regulation by miRNAs promotes the G1/S transition of the ES cell cycle enabling their rapid proliferation. Furthermore, our screening strategy provides an alternative and powerful approach for uncovering the role of individual miRNAs in biological processes as it overcomes the common problem of redundancy and saturation in the miRNA system.

ES cells are derived from the inner cell mass of the blastocyst and have been used successfully as a tool to understand molecular mechanisms of early mammalian development2. Because ES cells can undergo infinite and rapid self-renewal without compromising pluripotency, they hold great potential for regenerative medicine. However, rapid proliferation could be detrimental if it leads to uncontrolled cell growth following transplantation into the host. The rapid proliferation of ES cells is thought to be due to their unique cell cycle structure, especially their shortened G1 phase3,4. Insights into the cell cycle control of ES cells have been gained by investigating the expression of cell cycle proteins5-8. Furthermore, small RNAs have been implicated in ES cell proliferation based on the phenotype of *Dicer1* knockout ES cells9,10. More recently, using a *Dgcr8* knockout model, we reported a proliferation defect in ES cells specifically deficient in a subclass of small RNAs, the canonical miRNAs1. These miRNA-deficient cells showed a relative increase in the number of cells in the G1 phase of the cell cycle. This finding suggests that

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Author Contributions YW performed all experiments except Fig. 3a (JEB). SB developed and provided miRNA mimic library. AS built mutant reporters for Fig. 4. LB provided technical support. YW and RB designed all experiments, analyzed data and wrote the manuscript.

miRNAs normally suppress inhibitors of the G1/S transition allowing the rapid transition from mitosis to S phase. However, confirmation of this hypothesis requires the identification of the specific miRNAs and targets involved in the process. Identification of individual miRNA function is complicated by the fact that miRNAs often function redundantly and exist at saturating levels in a wild-type background. To overcome this difficulty, we designed a screening strategy where individual miRNA mimics were reintroduced into an otherwise miRNA deficient background (*Dgcr8* knockout ES cells) and then evaluated for rescue of the proliferation and cell cycle defects (Fig. 1a).

To evaluate the efficiency at which miRNA mimics could be transfected and function in *Dgcr8* knockout ES cells, we used a pool of five different siRNAs to knock down ubiquitous enhanced green fluorescent protein (eGFP) expression in the *Dgcr8* null background. The transfected siRNAs were able to knock down eGFP in greater than 80% of the ES cells as revealed by flow cytometry (Supplementary Fig. 1a). This finding indicates high transfection and loading efficiency of small RNAs into the RNA-induced silencing complex (RISC) in the *Dgcr8* knockout ES cells. These siRNAs suppressed cell growth when transfected into *Dgcr8* knockout versus wild-type ES cells, even when each siRNA was introduced individually (Supplementary Fig. 1b). This detrimental growth effect may be due to enhanced off-target effects of small RNAs in an otherwise global miRNA-deficient background. Transfection of the miRNA, miR-1, did not suppress growth in the *Dgcr8* knockout cells, but was able to repress a miR-1 reporter (Supplementary Fig. 1c and 1d). Therefore, growth suppression is sequence dependent. An initial small-scale screen using miRNA mimics showed that some miRNAs could actually promote growth of the *Dgcr8* knockout ES cells, partially rescuing the proliferation defect in these cells (data not shown). Therefore, we expanded our screen to a library of 266 known mouse miRNAs (Fig. 1b). Fourteen of these miRNAs dramatically improved *Dgcr8* knockout ES cell proliferation with a Z score >3 (P value < 0.001) (Fig. 1c **and** Supplementary Table 1). Intriguingly, 11 of these miRNAs shared a similar seed sequence (Fig. 1d), suggesting that they may regulate common targets. These data showed that our screening approach could identify miRNAs and even a common seed sequence that can promote cell proliferation.

To verify the proliferation promoting function of these miRNAs, the miRNA mimics were re-synthesized and re-tested in *Dgcr8* knockout ES cells. MiR-33 was not re-tested as it is not expressed at significant levels in ES cells11. All except one miRNA (miR-223) were verified to rescue proliferation in the knockout background (Fig. 2a). Combinations of the miRNAs did not further enhance proliferation (Supplementary Fig. 2). Importantly, transfection of the miRNAs into wild-type ES cells had no effect on proliferation suggesting that they already exist at saturating levels in these cells (Supplementary Fig. 3a). Furthermore, when inhibitors to these miRNAs were introduced individually or in combination, they minimally decreased proliferation in the wild-type background suggesting that this large family of miRNAs is functionally redundant (Supplementary Fig. 3b). miR-294 and −295 were also tested in *Dicer* knockout ES cell background, which showed similar results to *Dgcr8* knockout cells (Supplementary Fig. 4). In summary, our screening approach identified multiple miRNAs that promote ES cell proliferation. These miRNAs

share a similar seed sequence, act redundantly, and together exist at saturating levels in this functional role.

Previous reports have shown that four of the verified miRNAs (miR-291a-3p, 291b-3p, 294, and 295) are highly expressed in undifferentiated mouse ES cells and rapidly downregulated upon differentiation11-14. Indeed, the mir-290 cluster consists of the most highly expressed miRNAs in ES cells15. Therefore, we chose to focus further analysis on these four miRNAs plus miR-320, which is highly expressed in human ES cells (Ref 14). We evaluated the effect of these miRNAs on the cell cycle based on our previous finding that *Dgcr8* knockout ES cells accumulate in the G1 phase of the cell cycle. All five miRNAs were able to reduce the fraction of cells in G1 with a concomitant increase in the fraction of cells in S or G2/M phase (Fig. 2b). The most dramatic effects were seen for miR-291a-3p, miR-294 and miR-295, where the fraction of cells in G1 phase was similar to wild-type ES cells. These data show that ES cell specific miRNAs promote ES cell proliferation, at least in part by promoting the transition of cells from G1 to S phase. Therefore, we coin these miRNAs, ESCC miRNAs, for ES cell specific Cell Cycle regulating miRNAs.

The G1/S transition in cells is regulated by two cyclin/Cdk complexes: cyclin D/Cdk4,6 and cyclin E/Cdk216,17. In ES cells, the cyclin D/Cdk4,6 complex is not present, while the cyclin E/Cdk2 complex is present and constitutively active4,18. MiRNAs function as repressors of translation by binding the 3'UTR of their target mRNAs19-21. Therefore, we hypothesized that the ESCC miRNAs function by suppressing an inhibitor of the cyclinE/Cdk2 complex. *Cdkn1a* (*p21*) and *Cdkn1b* (*p27*) are inhibitors of the cyclin E/Cdk2 complex17. Furthermore, *p21* has been reported to be post-transcriptionally regulated during ES cell differentiation5 and the computational program, Targetscan22, identifies *p21* as a potential target for all five of the ESCC miRNAs. Therefore, we set out to determine whether *p21* is a true target of the ESCC miRNAs. First, we evaluated whether *p21* is upregulated in the *Dgcr8* knockout cells. Indeed, both mRNA and protein levels were dramatically increased (Fig. 3). Next, we reintroduced the ESCC miRNAs into *Dgcr8* knockout ES cells and evaluated their effects on *p21* levels. Interestingly, while mRNA levels showed a variable response to the ESCC miRNAs ranging from decreased to even elevated levels, the P21 protein was consistently and substantially downregulated confirming a predominantly post-transcriptional role for these miRNAs (Fig. 3). miR-294 and miR-295 showed the most dramatic effects (∼3 fold downregulation). These findings are consistent with the miRNA regulation of P21 translation.

It remains plausible that *p21* is an indirect target of the miRNAs or its upregulation is simply a consequence of the increased number of cells in G1. To determine whether *p21* is a direct target of the ESCC miRNAs, we constructed a luciferase reporter containing the 3'UTR of *p21* cloned downstream of a constitutively expressed firefly luciferase (Fig. 4a). Luciferase activity was inhibited by ∼3 fold in wild-type ES cells compared to *Dgcr8* knockout ES cells, consistent with the fold reduction of endogenous P21 protein expression (Fig. 4b). Targetscan 4.0 predicts that two sites in the 3'UTR of *p21* are bound by the ESCC miRNAs (Fig. 4a). Luciferase activity increased in wild-type cells when either of these two sites was mutated (Fig. 4b). The effect was further enhanced when both sites were mutated suggesting that these sites act independently (1.6 and 1.4 fold increase individually and 2.3 fold

increase together). However, mutation of both sites did not increase luciferase levels in wild type ES cells to that seen in *Dgcr8* Δ/Δ cells (Fig. 4b), suggesting other potential miRNA target sites in the 3'UTR. Consistent with this hypothesis, closer evaluation of the 3'UTR revealed other sites partially complementary to the seed sequence of ESCC miRNAs (Supplementary Fig. 5). To confirm that regulation of the *p21* 3'UTR is due to the ESCC miRNAs, individual ESCC miRNAs were cotransfected with the luciferase reporter. All ESCC miRNAs significantly inhibited luciferase expression, while the mock transfection and cotransfection with miR-1 did not (Fig. 4c). Furthermore mutating site 1 or site 2 diminishes repression, although not to control levels, again consistent with additional target sites for these miRNAs in the 3'UTR (Fig. 4d). These findings show that the ESCC miRNAs directly target the 3'UTR of the cell cycle inhibitor, *p21*.

To determine the functional role of *p21* in the cell cycle phenotype, we performed knockdown and overexpression experiments in *Dgcr8* knockout and wild-type cells respectively. Four different siRNA sequences against *p21* were tested, but all had toxic effects in the *Dgcr8* knockout background similar to that found with GFP siRNAs (Supplementary Figure 1 **and data not shown**). Again, this may be due to enhanced off-target effects in the knockout background. Overexpression of *p21* in wild-type ES cells was achieved using an EF1a promoter driving the *p21* ORF linked to an IRES-Puromycin-resistance cassette. Stable transfection of this overexpression construct lead to p21 protein levels 6−9 fold greater than wild-type levels (Supplementary Fig. 6a). Throughout this range of overexpression, the cells consistently showed an increased G1 fraction very similar to that seen in *Dgcr8* knockout cells (Fig. 4e). These results are consistent with *p21* being an important functional target of the ESCC miRNAs. Individual miRNAs are believed to target multiple downstream mRNAs rather than following a simple linear pathway23. To determine whether other G1/S regulators may be suppressed by miRNAs in ES cells, we performed mRNA microarray analysis on wild-type and *Dgcr8* knockout cells. Suprisingly, fewer than 2% of the total mRNAs were upregulated more than 2 fold in the knockout cells (data not shown). However, five members of the CyclinE/CDK2 regulatory pathway (*Cdkn1a, Rb1, Rbl1, Rbl2, Lats2*) were significantly increased in the knockout cells (Fig. 5a). All five are inhibitors of this pathway16,17,24. qRT-PCR confirmed increased expression of these genes (Fig. 5b). Furthermore, Targetscan predicts target sites of the ESCC miRNAs in the 3'UTRs of all five inhibitors (Fig. 5c). In contrast, one inhibitor of the Cyclin E/Cdk2 pathway, *Cdkn1b* (*p27*), was not significantly upregulated in the knockout cells and is not predicted to be a target of the ESCC miRNAs. Luciferase reporter assays confirmed miRNA based inhibition by the 3'UTRs of *Rbl2* and *Lats2* (Fig.5c). Inhibition of these 3'UTRs was phenocopied by the introduction of miR-294 into *Dgcr8* knockout cells (Supplementary Fig. 6b). Together, these data suggest that the ESCC miRNAs act at multiple levels of the CyclinE/Cdk2 pathway to promote the transition of ES cells from G1 to S phase (Fig. 5d).

Our results show that ES cell specific miRNAs play a central role in expediting the G1/S transition and promoting cellular proliferation. A shortened cell cycle is critical during early mammalian embryogenesis enabling the young embryo to rapidly grow prior to somatic differentiation. In contrast, an inappropriately shortened cell cycle in more differentiated

cells would likely result in abnormal cell growth with associated tumor formation26. Therefore, unraveling the mechanisms that promote the G1/S transition in mammalian embryonic cells is critical to understanding both normal embryonic development and cancer. We have previously shown that *Dgcr8* is required for ES cell differentiation as well as normal proliferation1. An interesting possibility is that these two phenotypes are linked. However, the ESCC miRNAs do not rescue the differentiation defects (Collin Melton and RHB, unpublished data) suggesting different miRNAs underlie the two phenotypes. The degree of redundancy we find for miRNA regulation both in terms of the number of miRNAs as well as the number of targets within the CyclinE/Cdk2 pathway is impressive, emphasizing the importance of hastening the G1/S transition in early embryonic cells. Furthermore, two recent studies showed that the ESCC miRNAs also regulate *de novo* methylation by targeting Rbl227,28, indicating multiple functions of these important miRNAs. Our paper presents a novel screening approach that overcomes issues of redundancy and saturation that is inherent to the miRNA system and, therefore, can be generally used to evaluate multiple functions of miRNAs in a wide array of biological processes.

METHODS

Tissue culture, transfection and miRNA screening

ES cells were cultured as previously described1. For the miRNA screening, *Dgcr8* Δ/Δ ES cells were plated at 4000 cells per well in a 96-well plate and grown for ∼24 hours prior to transfection. Transfection of small RNAs was performed using Dharmafect (Dharmacon, Thermo Fisher) based on manufacture's protocol. Mouse mi*RIDIAN* miRNA Mimic Library (Dharmacon, Thermo Fisher) was used for the screening. The final concentration of small RNAs was 100 nM unless specified. Twenty-four hours after transfection, media was replaced with fresh ES cell media. Cells were grown for another 3 days with media changes each day prior to the MTT assay. The MTT assay was performed as previously described1. Z scores were calculated using the robust Z-score analysis. The equation used is (X-median)/MAD. “X” is the growth rate for cells transfected with individual miRNAs. “Median” is the median of the growth rate of all transfected cells. MAD is the median absolute deviation. Similar results were obtained at 10 nM concentrations of the miRNA mimics, although the proliferation promoting effect of some miRNAs was slightly reduced (data not shown). Furthermore, dose curve for miR-294 shows that as little as 1.25nM still has significant proliferation promoting effects (Supplementary Fig. 2b). For experiments in Supplementary Figure 3, MTT assay was done as described above except 2,000 wild type ES cells per well were plated in a 96-well plate. Wild-type ES cells were plated at half the number of *Dgcr8* knockout cells on Day 0 so that roughly equal numbers of cells were present on the day of transfection (Day 1). This was also necessary to ensure wild type ES cells were not overly confluent at the time of the MTT assay.

Cell Cycle, QRT-PCR and Affymetrix Arrays

For the cell cycle and qPCR analysis, 50,000 *Dgcr8* Δ/Δ ES cells or 25,000 wild type ES cells per well were plated in a 12-well plate and grown for ∼24 hours prior to transfection. Each well of cells were split to 2 wells of a 12-well plate 24 hours after transfection with

synthetic miRNA mimics. After growing for another 24 hours, one well of cells was collected for cell cycle analysis, while the other well of cells was used for RNA extraction. Cell cycle analysis and QRT-PCR for mRNAs were performed as previously described1. Sequences of qPCR primers are listed in Supplementary table 2. Affymetrix Mouse Gene 1.0 ST arrays were probed by the Gladstone Genomics Core (www.gladstone.ucsf.edu/gladstone/site/genomicscore). Three biological samples were assayed for each cell line. Data was analyzed by Affymetrix Expression Console software. The Robust Multichip Analysis (RMA) algorithm was used to normalize the array signal data across chips.

Immunoblot analysis

0.3 million *DGCR8* Δ/Δ ES cells or 0.15 million wild type ES cells per well were plated in a 6-well plate and grown for ∼24 hours prior to transfection. Proteins were extracted using EBC buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA) containing 1X protease inhibitor cocktail (Roche) 24 hour post transfection. Around one third of the total cell lysates was resolved on a 12% SDS-PAGE. The proteins were then transferred to a PVDF membrane (Biorad) and processed for immunodetection. Blots were analyzed using ImageJ software (http://rsb.info.nih.gov/ij).

Luciferase reporter constructs

To construct the luciferase reporter with wild type *p21* 3'UTR, the 3'UTR of *p21* was cloned by PCR from the cDNA of reverse transcribed wild type ES cell mRNA. PCR products were subcloned to pCR2.1-Topo vector (Invitrogen) and sequenced. The 3'UTR was then cloned into the XbaI site of pGL3-control vector (Promega). To construct luciferase reporters with mutations, PCR was first performed using two sets of primers to generate two fragments of 3'UTR overlapped at mutation sites. These two fragments were then annealed and used as templates to amplify mutated 3'UTR. PCR products with mutations were then processed as above to make mutant luciferase reporters. Reporter for 3' UTRs of other mRNA was constructed using similar approach as above. Sequences of primers are listed in Supplementary Table 2.

Luciferase reporter assay

16,000 *Dgcr8* Δ/Δ ES cells or 8,000 wild type ES cells per well were plated in a 96-well plate and grown for at least 16 hours prior to transfection. For the transfection experiments, a mixture of 160 ng of reporter and 40 ng of control vector pRL-TK (Promega) were transfected using 1 μl Fugene-6 (Roche) to each well. Cells were lysed 12−16 hours later and processed for luciferase assay using Dual-Luciferase® Reporter Assay System (Promega). Luciferase activity was measured by Mithras LB 940 (Berthold Technologies). For the cotransfection with miRNAs, miRNAs were transfected using Dharmafect (Dharmacon, Thermo Fisher) ∼10 hours before transfecting the luciferase reporters.

Overexpression of Cdkn1a

0.5 millions of cells were plated in one well of a 6-well plate ∼24 hour prior to transfection. Cells were transfected with 5 μg plasmids. Cdkn1a or GFP was under control of E2F1α promoter and upstream of IRES-Pac (puromycin resistance). Cells were then selected at 0.6

μg/ml puromycin for at least 12 days before cell cycle and Western analysis. After selection, fraction of GFP positive cells is typically more than 95%. To prepare samples for cell cycle and Western analysis, 0.5 millions of cells were plated in one well of a 6-well plate and grew for ∼24 hours.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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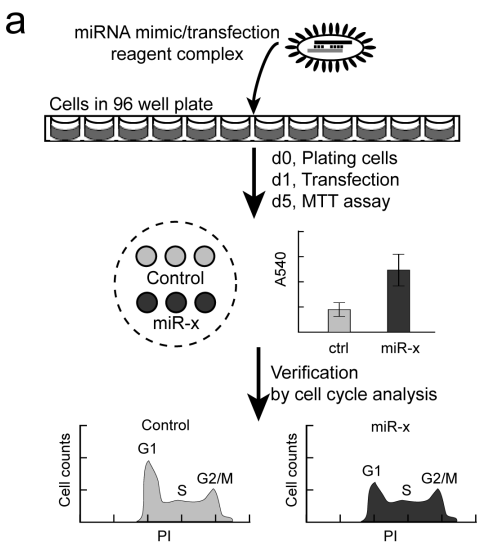

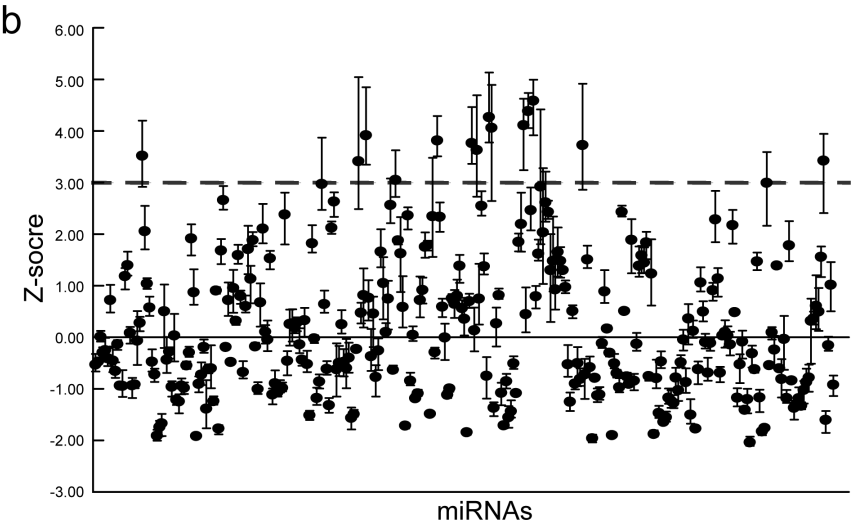

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miRNAs	Relative growth rate	Z-score
miR-19a	1.37	3.41
miR-20a	1.50	3.92
miR-20b	1.28	3.05
miR-33	1.45	3.73
miR-93	1.37	3.43
miR-106a	1.40	3.52
miR-223	1.47	3.81
miR-291a-3p	1.46	3.76
miR-291b-3p	1.42	3.63
miR-294	1.58	4.27
miR-295	1.53	4.06
miR-302b	1.54	4.11
miR-302c	1.61	4.39
miR-302d	1.66	4.59

d

miR-20a	UAAAGUGCUUAUAGUGCAGGUAG
miR-20b	CAAAGUGCUCAUAGUGCAGGUA
miR-93	CAAAGUGCUGUUCGUGCAGGUAG
miR-106a	CAAAGUGCUAACAGUGCAGGUA
miR-291a-3p	AAAGUGCUUCCACUUUGUGUGCC
miR-291b-3p	AAAGUGCAUCCAUUUUGUUUGUC
miR-294	AAAGUGCUUCCCUUUUGUGUGU
miR-295	AAAGUGCUACUACUUUUGAGUCU
miR-302b	UAAGUGCUUCCAUGUUUUAGUAG
miR-302c	CAAGUGCUUCCAUGUUUCAGUGG
miR-302d	UAAGUGCUUCCAUGUUUGAGUGU

Figure 1.

Screening for miRNAs that rescue the proliferation defects of *Dgcr8* Δ/Δ ES cells. (**a**) Screening strategy. Proliferation of ES cells transfected with individual miRNA mimics was first evaluated by the MTT assay. The positive hits were then assessed for their ability to rescue the G1 accumulation defects of *Dgcr8* Δ/Δ ES cells. (**b**) Z-scores for individual miRNA mimics. Shown are average Z-scores from triplicates. Error bar indicates the range of triplicates. (**c**) Top 14 positive hits with Z-score > 3 (P value < 0.001). The growth rate was normalized to mock transfected DGCR8 Δ/Δ ES cells. (**d**) 11 positive hits share similar seed sequence. Seed sequences are highlighted in gray box.

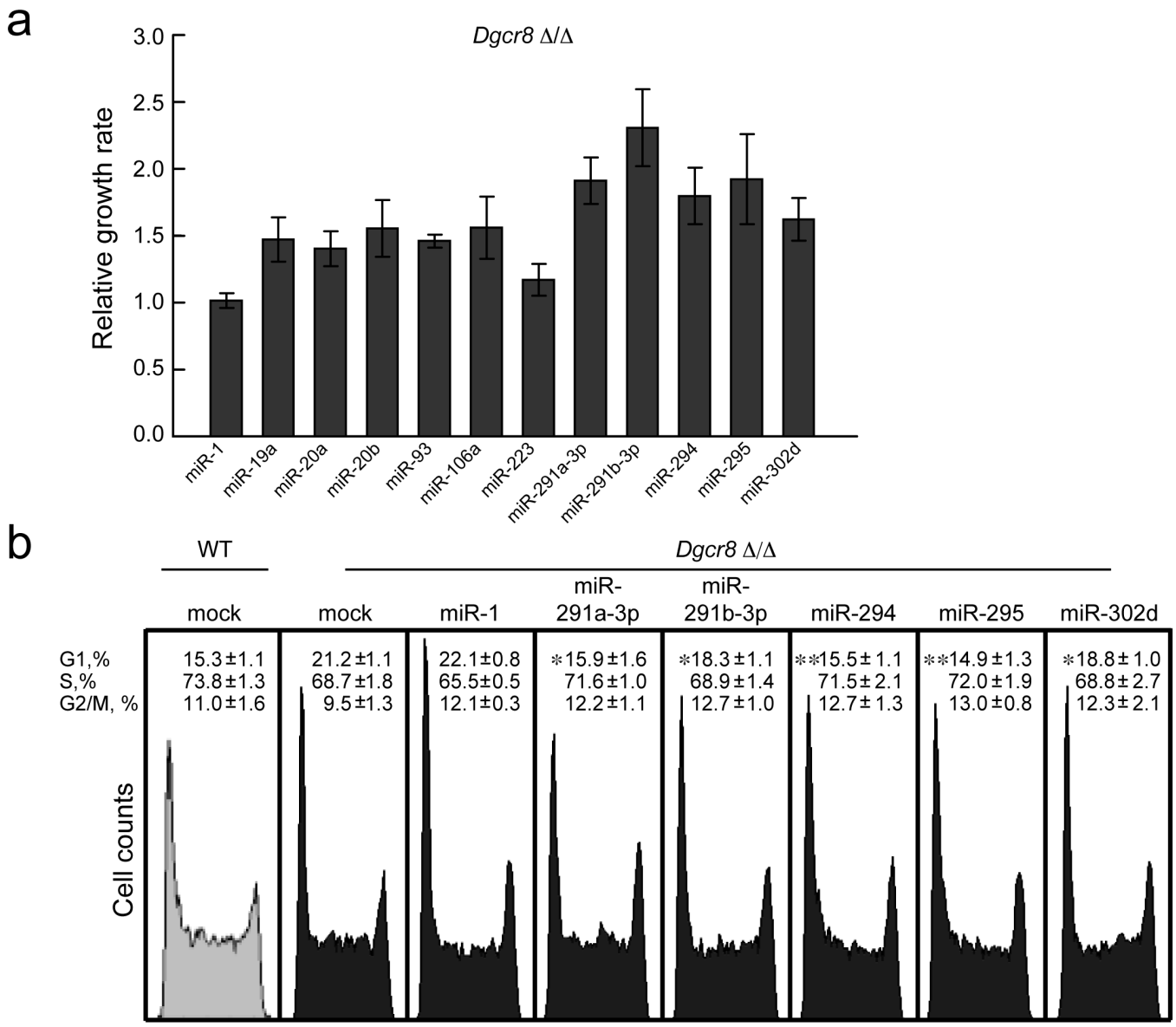

Figure 2.

Rescue of proliferation and G1 accumulation defects by representative miRNAs. (**a**) Resynthesized screen positive miRNAs promoted *Dgcr8* null ES cell proliferation. Growth rates were normalized to mock transfected *Dgcr8* Δ/Δ ES cells. miR-1 served as a control. Error bars represent s.d. n=6. (**b**) ES cell specific miRNAs rescued G1 accumulation of *Dgcr8* Δ/Δ ES cells. Shown is flow cytometry analysis of propidium iodide stained cells. Results are shown as means±s.d except for miR-1, for which is shown as mean±range. n=2 for miR-1. n=4 or 5 for rest of samples. *, P value < 0.01; **, P value < 0.001. The P value was calculated based on the 2-tailed t-test.

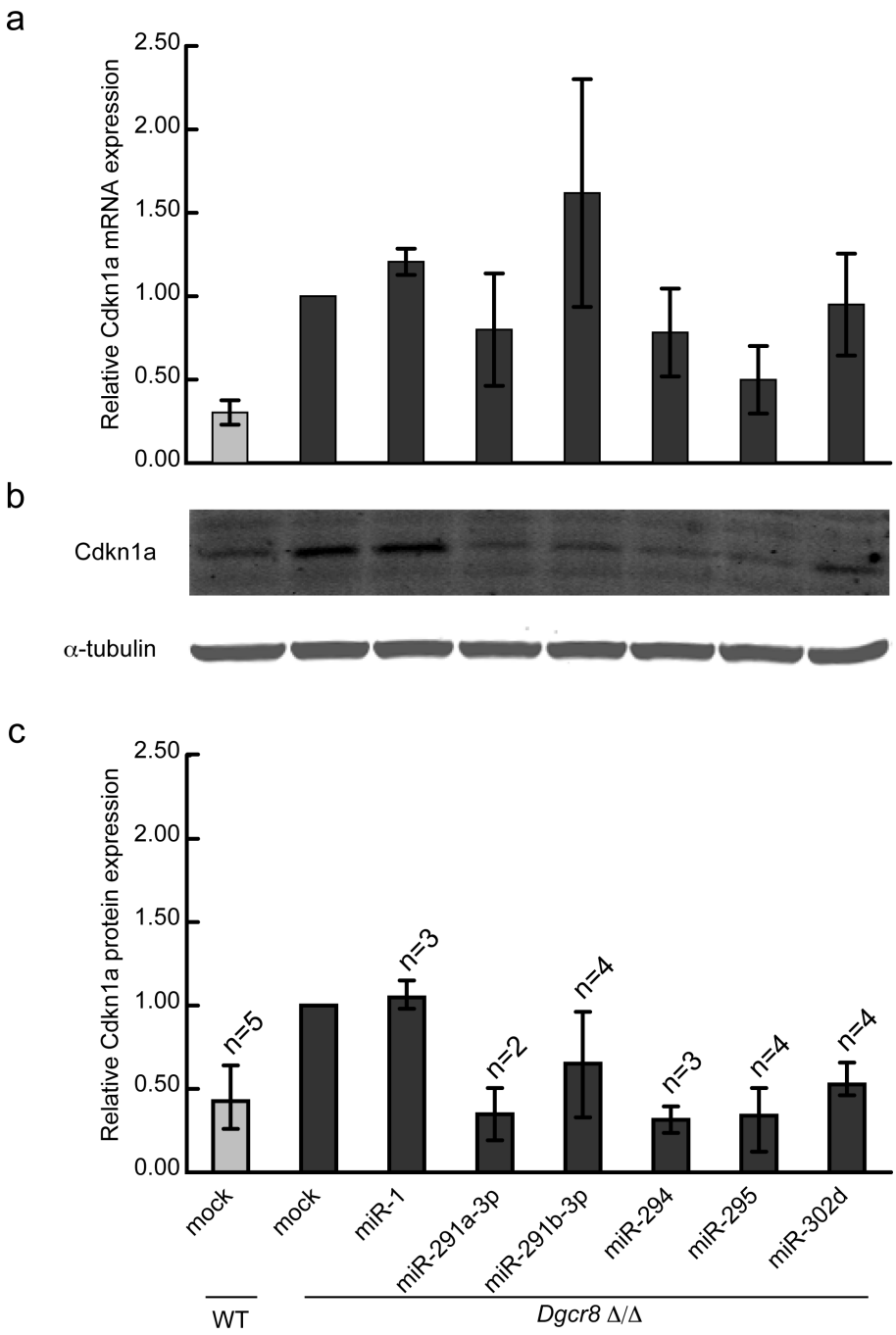

Figure 3.

Expression of *Cdkn1a* mRNA and protein upon introduction of ESCC miRNAs. (**a**) Quantatitive PCR. mRNA expression was normalized to mock transfected *Dgcr8* Δ/Δ ES cells. Error bars indicate s.d except for miR-1, for which the error bar indicates range. n=2 for miR-1. n=3 for wild type and n=4 for ESCC miRNAs. (**b**) Representative Western blot of *Cdkn1a* protein. (**c**) Densitometry for Western analysis of *Cdkn1a* protein. Data was normalized to mock transfected *Dgcr8* Δ/Δ ES cells. Numbers of independent transfection experiments performed are indicated on the top of each bar. Error bars indicate the range.

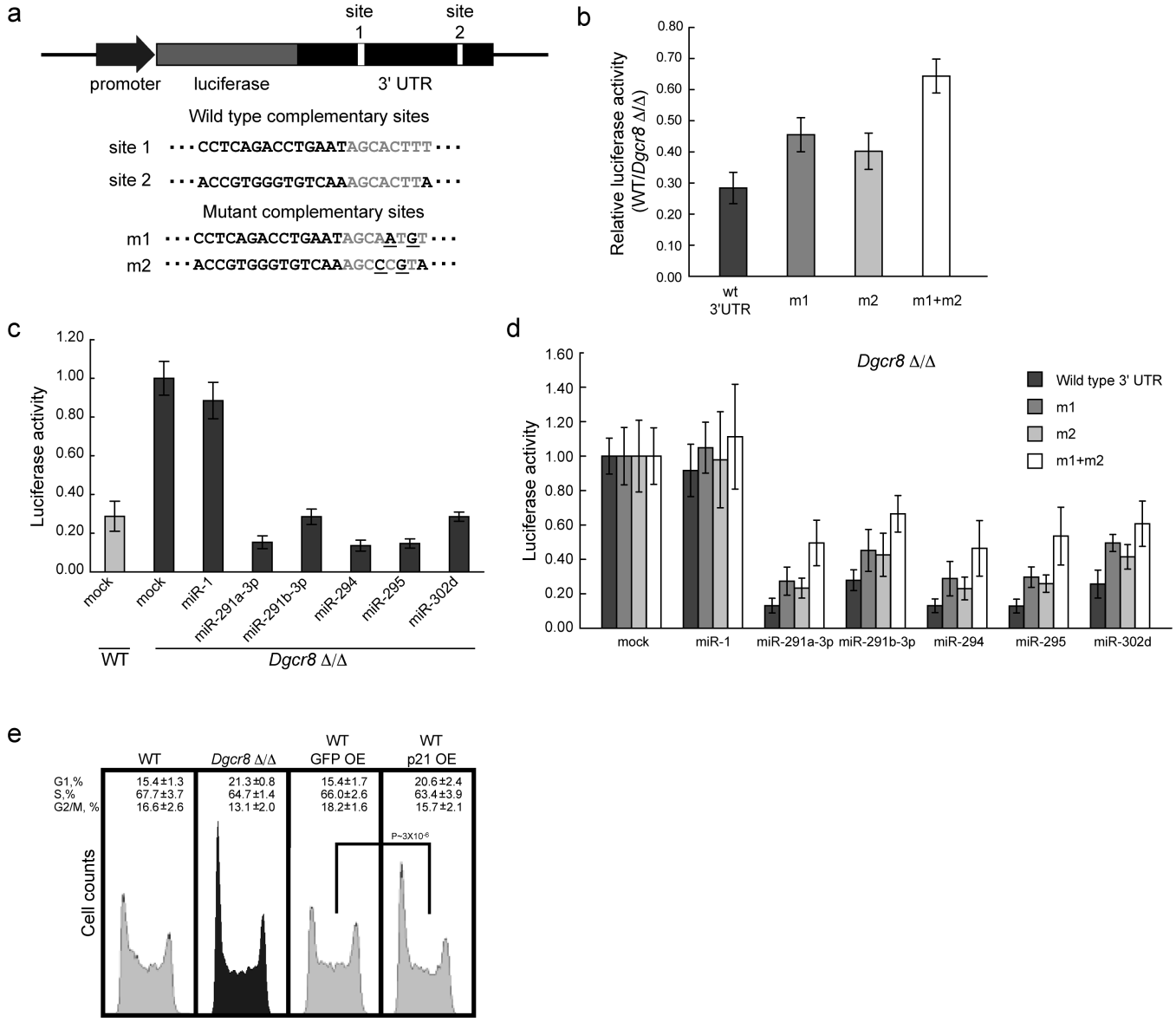

Figure 4.

Luciferase reporter assay indicates that ESCC miRNAs directly interact with 3' UTR of *Cdkn1a*. (**a**) Firefly luciferase reporter constructs. The 3' UTR of *Cdkn1a* (∼1.3 kb) was cloned downstream of firefly in the pGL3-control vector. Two predicted targeting sites and the corresponding mutations are listed. All experiments were normalized to cotransfected renilla luciferase. (**b**) Relative luciferase activity between wild-type and *Dgcr8* knockout cells following transfection of wild-type and mutant reporters. Error bars indicate s.d. n=8. (**c**) Cotransfection of wild-type reporter with ESCC miRNAs. Data was normalized to miRNA mock transfected *Dgcr8* Δ/Δ ES cells. Error bars indicate s.d. n=4 for wild type ES cells; n=8 for *Dgcr8* Δ/Δ ES cells. (**d**) Cotransfection of ESCC miRNAs with mutant reporter constructs. Each reporter was normalized to miRNA mock transfected *Dgcr8* Δ/Δ ES cells. Error bars indicate s.d. n=4. (**e**) Cell cycle profile of wild type ES cells with ectopic

expression of Cdkn1a. n=6 for wild type and *Dgcr8* Δ/Δ ES cells. n=12 (from 3 independent transfections) for wild type ES cells with GFP or Cdkn1a overexpression.

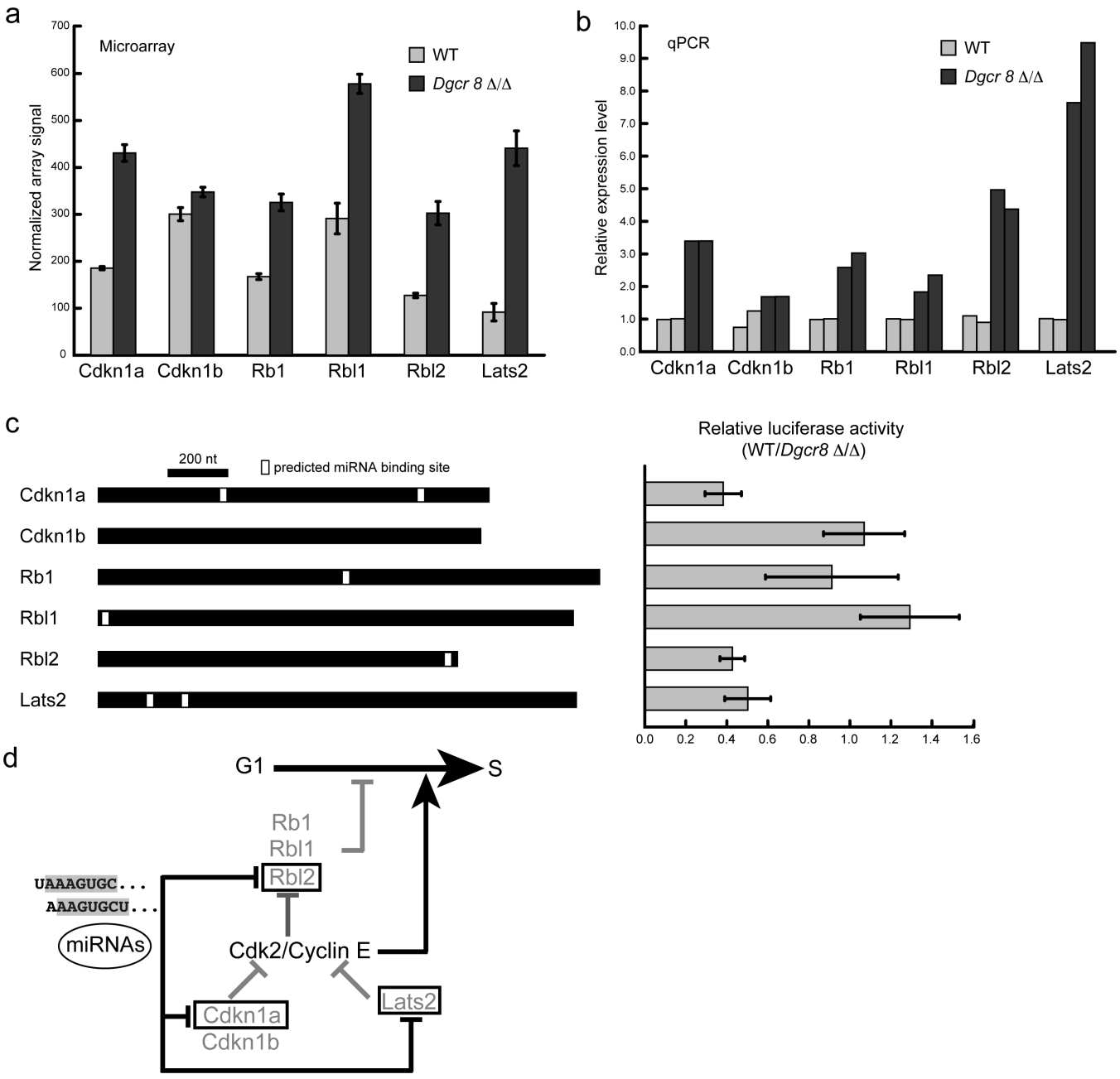

Figure 5.

Additional inhibitors of the Cyclin E/Cdk2 are regulated by miRNAs in ES cells. (**a**) Affymetrix Mouse Gene 1.0 ST arrays were probed with RNA from wild-type and *Dgcr8* Δ/Δ ES cells. Shown are the RMA normalized array signals for six inhibitors of the Cyclin E/Cdk2 induced transition from G1 to S. n=3. (**b**) qRT-PCR confirmation of array data. Shown is the relative expression of each gene to the average of wild-type samples. Each represents the average of a single biological replicate done in triplicate. (**c**) Diagrammatic representation of Targetscan predicted 3'UTR sites for the ESCC miRNAs and relative luciferase activity between wild-type and *Dgcr8* knockout cells following transfection of

respective reporters. Error bars indicate s.d. n=8. (**d**) Diagram of Cyclin E/CdK2 pathway with inhibitors regulated by ESCC miRNAs boxed.