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Xiaojing Yang, Min Feng, Xia Jiang, et al.

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RESEARCH COMMUNICATION

miR-449a and *miR-449b* are direct transcriptional targets of E2F1 and negatively regulate pRb–E2F1 activity through a feedback loop by targeting *CDK6* and *CDC25A*

Xiaojing Yang,[1,3] Min Feng,[1,3] Xia Jiang,[1] Zhenlong Wu,[1] Zhimei Li,[1] Meiyee Aau,[1] and Qiang Yu[1,2,4]

[1]Cancer Biology and Pharmacology, Genome Institute of Singapore, A*STAR (Agency for Science, Technology, and Research), Singapore 138672; [2]Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597

The Rb–E2F pathway drives cell cycle progression and cell proliferation, and the molecular strategies safeguarding its activity are not fully understood. Here we report that E2F1 directly transactivates *miR-449a/b*. *miR-449a/b* targets and inhibits oncogenic *CDK6* and *CDC25A*, resulting in pRb dephosphorylation and cell cycle arrest at G1 phase, revealing a negative feedback regulation of the pRb–E2F1 pathway. Moreover, *miR-449*a/b expression in cancer cells is epigenetically repressed through histone H3 Lys27 trimethylation, and epigenetic drug treatment targeting histone methylation results in strong induction of *miR-449a/b*. Our study reveals a tumor suppressor function of *miR-449a/b* through regulating Rb/E2F1 activity, and suggests that escape from this regulation through an aberrant epigenetic event contributes to E2F1 deregulation and unrestricted proliferation in human cancer.

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E2Fs transcriptional activity plays pivotal roles in cell cycle progression that is controlled by pRB through cell cycle-dependent phosphorylation (Trimarchi and Lees 2002). In response to mitogenic signaling, pRB is sequentially phosphorylated by the CDK complexes cyclin D–CDK4/6 and cyclin E–CDK2, leading to activation of E2F-responsive genes to promote cell cycle progression. In human cancer, the pRB-mediated repression of E2F is often disrupted through either genetic mutations that inactivate the *RB1* gene itself, overactivation of cyclin D–CDK4/6 kinases, or inactivation of the CDK inhibitor *p16* (Sherr and McCormick 2002). These changes result in the inappropriate release of E2F, thereby inducing transcriptional activation of E2F target genes and, consequently, cell proliferation.

The mammalian genome contains several hundred microRNAs (miRNAs) that are noncoding RNAs 18–25 nucleotides (nt) in length and that regulate the expression of 30% of human genes by either inhibiting mRNA translation or inducing its degradation (Ambros 2004; Lewis et al. 2005). It has become evident that miRNAs regulate a variety of biological processes, and their expression is often deregulated in human malignancy (Lu et al. 2005; Esquela-Kerscher and Slack 2006; Volinia et al. 2006). On the one hand, miRNAs have roles in tumorigenesis by modulating oncogenic and tumor suppressor pathways, such as p53, Myc, E2F1, Ras, and BCR-ABL (Ventura and Jacks 2009). On the other hand, their expression can be regulated by several oncogenic or tumor suppressor transcription factors, including E2Fs (O'Donnell et al. 2005; Chang et al. 2007; He et al. 2007; Raver-Shapira et al. 2007). Intriguingly, a number of E2F-up-regulated miRNAs, such as *miR-106b* and *miR-17-92* cluster, can in turn act as negative feedback regulators of the E2F1 pathway by directly targeting and inhibiting *E2F1* expression, revealing a potential mechanism of miRNA in maintaining cellular homeostasis (O'Donnell et al. 2005; Sylvestre et al. 2007; Petrocca et al. 2008; Pickering et al. 2009).

Here we report the discovery of the miRNAs *miR-449a/b* as direct transcriptional targets of E2F1. Unlike other E2F-regualted miRNAs that directly target and affect E2F1 expression, *miR-449a/b* negatively regulate the E2F1 activity by targeting CDK6 and CDC25A toward pRb phosphorylation, providing a distinct fail-safe feedback mechanism controlling E2F1 activity. We found that *miR-449a/b* expression in cancer cells is epigenetically inactivated by the oncogenic trimethylated histone H3 Lys27 (H3K27me3), which can be reversed by epigenetic drug treatment targeting histone modifications. These findings reveal a feedback mechanism to guard against the excessive E2F1 activity and suggest that the epigenetic loss of *miR-449a/b* may represent a novel mechanism leading to E2F1 deregulation in human cancer.

Results and Discussion

miRNA profiling identifies miR-449a/b *as novel E2F1-inducible targets*

Several miRNAs have been implicated previously in E2F's network. To identify additional miRNAs potentially involved in E2F1 function, we examined the miRNA expression profile using an E2F1-inducible Saos-2 cell line. In this cell system, E2F1 is fused to the estrogen receptor (ER)-binding domain, and ER-E2F1 is activated by ER ligand 4-OHT to induce the expression of bona fide E2F1 targets (Vigo et al. 1999), as validated by gene expression profiling (Supplemental Fig. S1A). In the same cells, we used the Agilent human miRNA microarray system that contains probes for 723 human miRNAs to profile miRNA expression, and three independent experiments show that only *miR-449a* and *miR-449b* are consistently and robustly induced following 4-OHT addition (Supplemental Table S1).The other E2F1-responsive miRNAs were only modestly induced, with the majority of induction for less than twofold. Notably, *miR-106b* and

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[3]**These authors contributed equally to this work.**
[4]**Corresponding author.**
E-MAIL yuq@gis.a-star.edu.sg; FAX 65-6478-9003.
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miR-20a, which have been reported previously to be E2F1 targets (Sylvestre et al. 2007; Woods et al. 2007; Petrocca et al. 2008), were not much affected by E2F1 in this system. This led us to pursue *miR-449a/b* for further studies, as they appear to be strong candidates regulated by E2F1 and potentially novel miRNA targets of E2F1.

Quantitative measurement of the mature form of the miRNA using a stem–loop real-time PCR (qPCR) assay validated the strong *miR-449a/b* induction in Saos-2 cells expressing ER-E2F1 following the addition of 4-OHT, but this induction was not seen in Saos-2 cells expressing the DNA-binding mutant ER-E132 cells (Fig. 1A). *miR-34a*, a known p53 target used here as a negative control, showed no response to 4-OHT. This effect of E2F1 on *miR-449a/b* is not exclusive to Saos-2 cells, as it was also observed in U2OS-ER-E2F1 cells but, again, not in their counterparts expressing the mutant ER-E132 (Fig. 1B). To determine the regulation of *miR-449a/b* by the endogenous E2F1, we synchronized Saos-2 in the G0/G1 phase by serum starvation. Cells entered the S phase when serum-starved cells were stimulated with serum at different time points (Fig. 1C). Orchestrating the enhanced expression of E2F1 target gene *CCNE1*, we detected the corresponding increase in *miR-449a/b*, but not *miR-34a*, as cells entered S phase over time (Fig. 1D). Similar results were also obtained in MCF10A cells (Supplemental Fig. S1B). Thus, *miR-449a/b* expression correlated well with the endogenous E2F1 activation during cell cycle progression. Taken together, these data support that *miR-449a/b* are transcriptional targets of E2F1.

E2F1 directly transactivates miR-449 *in parallel with its host gene,* CDC20B

miR-449a/b have been mapped to the first intron of *CDC20B* on chromosome 5 (Fig. 2A). Previous studies involving the genome-scale analysis of transcription factor binding and histone modifications predict that these miRNAs share a common promoter with their host gene, *CDC20B* (Marson et al. 2008; Ozsolak et al. 2008). This is further supported by the location of the histone H3K4me3, a histone mark indicative of core promoter region, as revealed by an H3K4me3 chromatin immunoprecipitation (ChIP)-seq genomic database (Supplemental Fig. S3A). Sequence analysis of the promoter region revealed two conserved E2F-binding sites (−225 to −208 and −137 to −120) at the core promoter region of *CDC20B/miR-449* (Fig. 2A). In agreement with this prediction, a quantitative RT–PCR (qRT–PCR) assay showed that *CDC20B* expression was also up-regulated by E2F1 (but not by the mutant E2F1) to a similar extent as *miR-449*, along with other canonical E2F1 targets, *CCNE1* and *CCNE2* (Fig. 2B). Notably, the seemingly much greater fold of induction on *CDC20B/miR-449*, as compared with other E2F1 targets, simply reflects a nearly undetectable basal level of transcription at this locus in Saos-2 cells. These results support the notion that the transcriptional unit, containing *CDC20B* and hosted *miR-449a/b*, is transcriptionally activated by E2F1.

To further demonstrate the regulation of E2F1 on the promoter of *CDC20B/miR-449*, we performed the luciferase

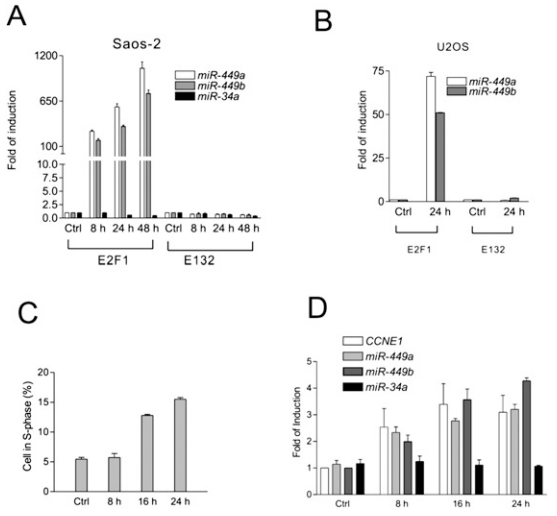

Figure 1. *miR-449a* and *miR-449b* are E2F1-inducible targets. (*A*) miRNA stem–loop qRT–PCR assays quantifying the expression changes of *miR-449a/b* and *miR-34a* in Saos-2-ER-E2F1 and Saos-2-ER-E132 cells treated with 4-OHT at indicated times. (*B*) The same as in *A* in U2OS cells expressing ER-E2F1 and ER-E132. (*C*) Saos-2 cells were serum-starved for 48 h followed by serum stimulation for the indicated times. Quantifications of cells in S phase were determined by FACS analysis. (*D*) qRT–PCR measurement of changes of expression levels of *CCNE1*, *miR-449a/b*, and *miR-34a*.

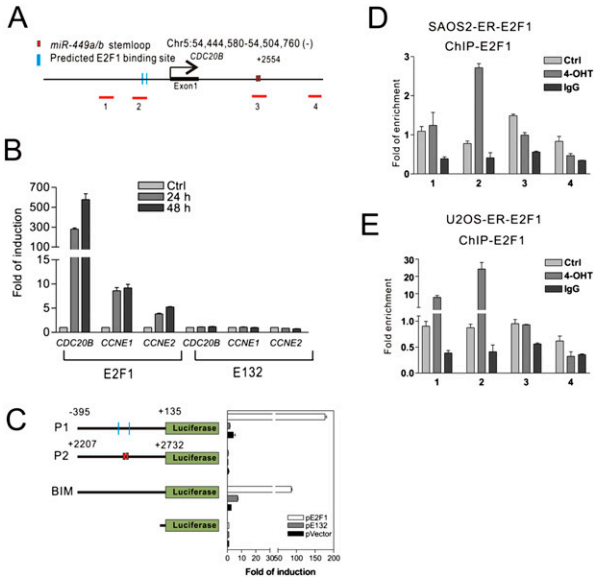

Figure 2. Direct transcriptional activation of *miR-449* by E2F1. (*A*) Schematic representation of *miR-449/CDC20 locus*: *miR-449*a/b is mapped to chromosome 5 within the first intron of *CDC20B*. Blue bars indicate two putative E2F1-binding sites, while red lines with numbers indicate the locations of ChIP-PCR primers used in *D* and *E*. (*B*) qRT–PCR of *CDC20B*, *CCNE1*, and *CCNE2* in Saos-2 cells expressing ER-E2F1 and ER-E132 following 4-OHT treatment for 24 and 48 h. (*C*) HCT116 cells were transfected with pcDNA4-basic, E2F1, and E132 (100 ng), together with a luciferase reporter construct containing the indicated genomic regions. The Bim reporter is used as a positive control here. Relative luciferase activities were measured 48 h after transfection. Results are depicted as fold induction, after normalization to Renilla luciferase activity. (*D*,*E*) ChIP-qPCR assays showing E2F1 binding to the *CDC20B/miR-449* core promoter region. Saos-2 ER-E2F1 and U2OS ER-E2F1 cells were treated with or without 4-OHT for 24 h before ChIP analysis using E2F1 or IgG antibody. Genomic DNA fragments covering an ~5.0-kb region surrounding the *miR-449/CDC20B* TSS as indicated in *A* were analyzed by qPCR.

reporter assay. An ~600-base-pair (bp) genomic fragment (P1) flanking the core promoter region was isolated and subcloned into a luciferase reporter plasmid. Another 500-bp DNA fragment (P2) that contains the immediate upstream region of the *miR-449* mature sequence was also included in the reporter assay as a control. The results show that the P1 construct containing the *CDC20B/miR-449* core promoter region responded strongly to the transfected wild-type E2F1, but not to the DNA-binding mutant E132 (Fig. 2C). This induction of promoter activity was comparable with a reporter for *BIM*, a known E2F1 target (Zhao et al. 2005), as a positive control. In contrast, the P2 construct showed no response to E2F1. Furthermore, to validate a direct binding of E2F1 to the promoter region, we conducted a ChIP assay in Saos-2 ER-E2F1 cells using the E2F1 antibody, coupled with qPCR. To this end, we used a series of PCR primers covering an ~5-kb genomic region surrounding the transcription start site (TSS), and the results detected a strong E2F1 enrichment in the core promoter region corresponding to the predicted E2F1-binding sites, but not in the immediate region upstream of *miR-449* (Fig. 2D). Similar results were also obtained in U2OS-ER-E2F1 cells (Fig. 2E). These data provide compelling evidence that *miR-449a/b* are direct transcriptional targets of E2F1.

miR-449a/b *directly target and inhibit* CDK6 *and* CDC25A

The TargetScan database (http://www.targetscan.org) predicts that *miR-449a* and *miR-449b* share the same target sequence and can modulate up to 400 genes. Given that E2F1 and its target genes are essential for S-phase entry, we predicted a functional role of *miR-449a/b* in such cell cycle regulation. Among the *miR-449* putative targets with a potential role in cell cycle regulation, we find that *CDK6* and *CDC25A* are of particular relevance given their established activities in G1/S-phase transition. The TargetScan algorithm predicts that the *CDK6* and *CDC25A* 3′ untranslated regions (UTRs) contain two *miR-449*-binding sites and one *miR-449*-binding site, or MREs (miRNA-responsive elements), respectively (Fig. 3A). The cyclin D–CDK4/6 complex is a central component of the G1/S-phase checkpoint by regulating the pRb–E2F1 pathway (Sherr and McCormick 2002). CDC25A, on the other hand, regulates pRb activity through activation of CDK2 by removing its inhibitory phosphorylation (Ray et al. 2007). Its frequent overexpression found in human cancers is associated with more aggressive cancer phenotypes with poor prognosis (Xu et al. 2003; Kang et al. 2008). Given the oncogenic roles of both CDK6 and CDC25A, it is tempting to speculate a tumor suppressor role of *miR-449* in enforcing E2F1 functions.

To experimentally validate that *miR-449* can target *CDK6* and *CDC25A*, we used multiple approaches to investigate how *miR449* may alter expression of these genes at the transcriptional or post-transcriptional level. As determined by both the quantitative Illumina gene expression (Fig. 3B) and the TaqMan assay (Supplemental Fig. S2A), activation of E2F1 by 4-OHT resulted in the down-regulation of *CDK6* and *CDC25A* mRNA. Notably, *CDK4* mRNA was also down-regulated following E2F1 activation. This change of *CDK4* might be indirect, as its 3′ UTR does not contain a *miR-449* target sequence and is unlikely to be a direct target of *miR-449*. Corresponding to the reduced mRNA levels, the down-regulation at their protein levels following E2F1 activation by OHT was also evident in both Saos-2 and U2OS cells, as determined by Western blotting (Fig. 3C).

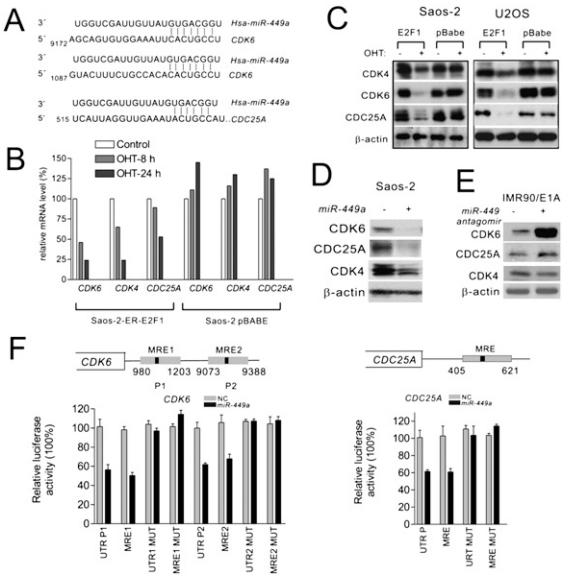

Figure 3. *miR-449a/b* directly target and inhibit *CDK6* and *CDC25A* protein expression. (*A*) Seed sequence base-pairing between *miR-449a/b* and MREs in the 3′ UTRs of *CDK6* and *CDC25A* predicted by TargetScan. Note that two *miR-449* target sites are predicted in the *CDK6* 3′ UTR. (*B*) Gene expression levels of indicated CDKs and CDC25A in Saos-2 ER-E2F1 cells and control pBabe cells before and after 4-OHT treatment for 24 h. (*C*) Western blot analysis of CDK4, CDK6, and CDC25A in Saos-2 and U2OS ER-E2F1 and control cells treated with 4-OHT. (*D*) Western blot analysis showing the protein level changes of CDK6, CDK4, and CDC25A in Saos-2 cells treated with 100 nM *miR-449a* mimics. (*E*) Western blot analysis of CDK6, CDC25A, and CDK4 in IMR90/E1A cells treated with 100 nM antagomir inhibitor of *miR-449*. (*F*) The *top* panel shows the two regions (P1 and P2) of the 3′ UTR of *CDK6* and one region of the 3′ UTR of *CDC25A*, as well as the short fragments containing the MREs or corresponding mutant on MRE sequences (UTR or MRE MUT) subcloned to a luciferase reporter. The *bottom* panel shows that *miR-449a* represses the luciferase reporter activity containing the 3′ UTR of *CDK6* or *CDC25A*, but not those containing the mutant MRE. All experiments were done at least in triplicate, twice ($n \geq 6$). Values were normalized to internal Renilla Luciferase control, and error bars denotes standard deviation.

To directly assess the effect of *miR-449* on *CDK6* and *CDC25A*, we transfected the *miR-449a* mimics into Saos-2 cells. Consistent with the endogenous induction of *miR-449*, transfection of *miR-449a* mimics at as low as 10 nM resulted in ablation of the protein levels of all three genes (Fig. 3D; Supplemental Fig. S2B). Moreover, using lung fibroblast IMR90 cells infected with E1A, which activates E2F pathway through inactivating Rb, we show that transfection with the antagomir inhibitor of *miR-449a/b* resulted in induction of both CDK6 and CDC25A protein levels (Fig. 3E), although CDK4 was not induced. These loss-of-function data, together with overexpression data, demonstrate the inhibitory effects of *miR-449* on CDK6 and CDC25A expression.

We next determined whether *miR-449* targets and inhibits the expression of *CDK6* and *CDC25A* through the 3′ UTR. To achieve this, we subcloned the portions of a *CDK6* or *CDC25A* 3′ UTR containing the predicted *miR-449* target sequences individually and also

the 23-nt-long MRE fragments to a luciferase reporter vector (Fig. 3F). *miR-449a* mimics, but not the control miRNA mimics, were able to reduce the luciferase activity in these reporter constructs by ~50%. However, the profound repression was abolished when the seed sequences of *miR-449* target sequences were mutated in either the constructs containing only the MRE fragment or in the constructs containing the 3′ UTR (Fig. 3F). These results provide strong evidence that *miR-449* targets and down-regulates *CDK6* and *CDC25A* through directly binding to their 3′ UTRs.

miR-449a/b *induce cell cycle arrest at G1 phase through inhibition of pRb–E2F1 activity*

In agreement with the synchronized down-regulation of CDK6 and CDK4 as well as a possible CDK2 inactivation upon inhibition of CDC25A protein expression, their major cellular targets, such as phosphorylated pRb, are expected to be down-regulated to a minimal level. Indeed, in several cancer cell lines examined, introduction of *miR-449a* mimics resulted in nearly complete inhibition of pRb phosphorylation and reduced E2F1 expression, as illustrated in breast cancer MCF-7, lung carcinoma H1299, and noncancerous breast epithelial MCF-10A cells (Fig. 4A). However, transfection of *miR-449a* in colon cancer DLD1 cells did not inhibit pRb phosphorylation and E2F1 expression, despite a dramatic drop of both CDK6 and CDC25A protein expression (data not shown). This suggests that the functional consequence of CDK6 and CDC25A inhibition is likely to be cell type-specific, and this could be due to frequent gene or epigenetic alterations accumulated along the pRb pathway in some cancer cells. *miR-449b*, which shares the same target sequence as *miR-449a*, gave rise to similar results (Supplemental Fig. S2C).

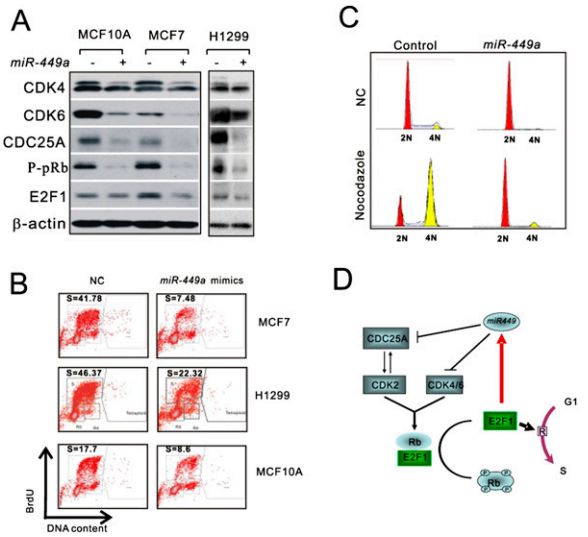

Figure 4. *miR-44a/b* regulate cell cycle G1/S progression. (*A*) Western blot analysis showing the changes of CDK6, CDC25A, p-Rb, and E2F1 in MCF10A, MCF-7, and H1299 cells treated with *miR-449a*. (*B*) S-phase index shown by BrdU incorporation assay in MCF-7, H1299, and MCF-10A cells treated with 100 nM control (NC) or *miR-449a* mimics. (*C*) MCF-7 cells were transfected with negative control (NC) and *miR-449a* mimics, followed by 200 ng/mL nocodazole treatments for 16 h, before cells were harvested for FACS analysis. (*D*) A proposed model of E2F1-*miR-449* circuitry as an autoregulatory negative feedback loop in cell cycle progression and growth control.

To confirm that these alterations on cell cycle regulators indeed conferred corresponding changes in cell cycle progression, we performed bromodeoxyuridine (BrdU) incorporation assays to measure the S-phase entry indicative of cell proliferation. As shown in Figure 4B, MCF7, H1299, and MCF10A cells transfected with *miR-449a* mimics exhibited a profound reduction of the BrdU proliferation index as compared with cells transfected with control miRNA mimics. Furthermore, MCF-7 cells treated with *miR-449a* mimics were completely arrested at G1 phase in the presence of nocodazole, a microtubule-depolymerizing drug that normally blocks cell cycle progression in G2/M (Fig. 4C). These results are consistent with a role of *miR-449* in inhibiting cell cycle progression at G1 phase by down-regulating CDK6 and CDC25A. Our findings uncover a pathway by which E2F1 induces expression of *miR-449*, which inhibits pRb phosphorylation by interfering with the stability or translation of its direct targets, *CDK6* and *CDC25A*, thereby providing a negative feedback loop mechanism to restrain E2F1 activity and cell proliferation (Fig. 4D).

Epigenetic repression of miR-449a/b *expression by histone modifications in cancer cells*

Several recent studies have indicated the down-regulation of *miR-449* in a variety of human malignancies, including lung, prostate cancers, and adrenal hyperplasia (Liang 2008; Iliopoulos et al. 2009; Noonan et al. 2009). In agreement with these reports, we found that *miR-449a/b* were expressed in much lower levels in a panel of examined cancer cell lines as compared with the noncancerous epithelial MCF10A cells (Fig. 5A). The mechanism by which *miR-449* is down-regulated in human cancer is currently unknown, but one possibility is that its down-regulation might be caused by aberrant epigenetic events. To test this hypothesis, we enquired a genome-wide mapping database of histone methylation marks in MCF-7 cells generated in-house using ChIP-Seq Solexa technology (ET Liu, pers. comm.). This high-resolution histone methylation map at the *CDC20B/miR-449* locus indicates a strong enrichment of both the repressive histone H3K27me3 mark and the activating H3K4me3 mark surrounding the TSS (Supplemental Fig. S3A). This indicates a typical "bivalent" histone modification state, which has been well characterized as a hallmark of gene repression in both embryonic stem cells and adult cancer cells (Azuara et al. 2006; Bernstein et al. 2006; Mikkelsen et al. 2007; Pan et al. 2007; Zhao et al. 2007). This result suggests that *miR-449a/b* expression might be epigenetically repressed by histone H3K27me3 in MCF-7 cells. Further ChIP-qPCR analysis confirmed the strong enrichment of both H3K27me3 and H3K4me3 in MCF-7 cells, but only H3K4me3 in MCF-10A cells (Fig. 5B), and this finding is consistent with the much lower expression of *miR-449* in MCF-7 as compared with MCF-10A cells. Furthermore, since DNA hypermethylation is now known to be mutually exclusive with H3K4me3 (Ooi et al. 2007; Weber et al. 2007), the presence of H3K4me3 suggests that DNA hypermethylation is unlikely to be involved in *miR-449* repression in MCF-7 cells.

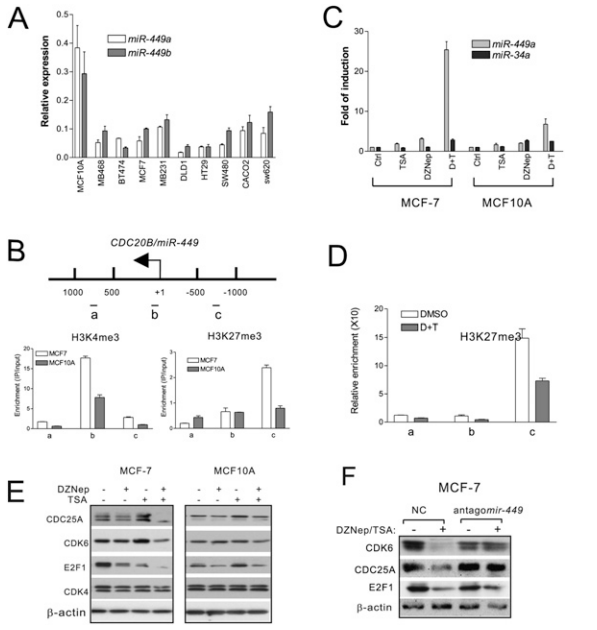

Figure 5. *miR-449a/b* are epigenetically repressed in cancer cells through histone methylation and are activated by epigenetic drugs targeting histone methylation. (*A*) qRT–PCR showing the differential expression levels of *miR-449a/b* in MCF10A and a series of cancer cell lines. (*B*) H3K27me3 and H3K4me3 ChIP analysis at *miR-449/CDC20B* locus in MCF-7 and MCF10A cells. The positions of examined DNA fragments are indicated by a, b, and c. (*C*) qRT–PCR of *miR-449a* and *miR-34a* in MCF-7 and MCF-10A cells treated with TSA (100 nM), DZNep (5 μM) alone, or in combination for 48 h. (*D*) ChIP-qPCR of H3K27me3 in MCF-7 following the D/T treatment. (*E*) Western blot analysis of indicated protein expression in MCF7 and MCF-10A cells treated as in *C*. (*F*) Western blot analysis of CDK6, CDC25A, and E2F1 in MCF-7 cells treated with DZNep/TSA for 48 h in the presence or absence of 100 nM antagomir inhibitor of *miR-449*.

We showed recently that the small-molecule histone methylation inhibitor Deazanoplasnocin A (DZNep) inhibits H3K27me3, and its combination with histone deactylase (HDAC) inhibitor trichostatin A (TSA) can effectively reverse gene repression associated with bivalent chromatin modifications (Tan et al. 2007; Jiang et al. 2008; Yang et al. 2009). Indeed, consistent with the different chromatin states in MCF-7 and MCF-10A cells, the combination treatment of DZNep and TSA, but not the single treatment, resulted in a dramatic induction of *miR-449* expression in MCF-7 cells, and this induction was much weaker in MCF-10A cells (Fig. 5C). In contrast, *miR-34a*, which contains no such histone modification in MCF-7 cells (data not shown), did not respond to this treatment. A similar result was also observed in colon cancer SW480 cells (Supplemental Fig. S3C). Consistent with the notion that *miR-449* is in the same transcriptional unit as *CDC20B*, we detected a similar level of induction of *CDC20B* following the same drug treatment (Supplemental Fig. S3B). Furthermore, DZNep/TSA treatment in MCF-7 cells effectively removed the H3K27me3 at the *CDC20B*/*miR-449* locus (Fig. 5D), providing an explanation for the strong induction of *miR-449* and *CDC20B* following the drug treatment.

Consistent with the induction of *miR-449*, the DZNep/TSA combination resulted in the synergistic reduction of protein levels of CDK6 and CDC25A, but not CDK4, as well as the down-regulation of pRb phosphorylation and E2F1 in MCF-7 and SW480 cells (Fig. 5E; Supplemental Fig. S3D), but not in MCF10A cells (Fig. 5E). Finally, cells treated with *miR-449* antagomir effectively rescued the drug-induced down-regulation of both CDK6 and CDC25A (Fig. 5F), validating the inhibitory effects of endogenous *miR-449* on CDK6 and CDC25A in this context. Our in vitro cell line evidence of epigenetic mechanism, together with previous reports in clinical primary tumor samples, strongly support the notion that the epigenetic repression of *miR-449* may be widely present within human cancer cells. We hypothesize that transcriptional inactivation of *miR-449* may be necessary to maintain the high E2F1 activity to promote transformation during tumorigenesis in certain contexts. In addition, it may also confer the elevated expression of CDC25A protein frequently seen in various types of human cancers. Furthermore, *miR-449* can target HDAC1 or Wnt1 for growth inhibition, as reported recently (Iliopoulos et al. 2009; Noonan et al. 2009). Therefore, *miR-449* might function as a tumor suppressor by inhibiting multiple oncogenic events, and pharmacologic reactivation of *miR-449* might have therapeutic benefits in human cancer.

Conclusions

Due to its pivotal and multifunctional role in cell cycle control, it is challenging to understand the paradoxical behavior of E2F1 as an oncogene or a tumor suppressor. Here we described the identification of *miR-449* as a novel E2F1-inducible target and verified its functional role in negatively regulating E2F activity through a feedback loop mechanism. Furthermore, the negative feedback effect of *miR-449* on E2F1, by targeting oncogenic CDK6 and CDC25A, is distinguishable from other miRNA targets of E2F1 that feedback E2F1 by directly decreasing E2F1 expression. Finally, the epigenetic inactivation of *miR-449* in tumor cells may disable this negative feedback regulation, therefore providing a proliferative advantage. Thus, restoration of *miR-449* expression through epigenetic drug treatment may result in inhibition of multiple oncogenic signaling in cancer cells. The results described here provide a plausible mechanism to expand our understanding of the E2F1 paradox in a certain context and its deregulation in human cancer.

Materials and methods

miRNA expression profiling and qPCR

The Agilent Human miRNA Microarray Kit version 2 (G4470B) was used to profile miRNA expression. TaqMan miRNA assays were used to quantify the level of mature miRNAs following the manufacturer's protocol (Applied Biosystems).

Transfections

miRIDIAN miRNA mimics and hairpin antagomirs were obtained from Dharmacon, and were transfected using lipofectamine RNAimax at a final concentration of 10–100 nM.

ChIP-PCR

ChIP-PCR assays were performed as described previously (Jiang et al. 2008) using E2F1 and histone mark-specific antibodies anti-E2F1 (C20X,

Santa Cruz Biotechnologies), anti-H3K27me3 (07-449, Upstate Biotechnologies), and anti-H3K4me3 (07-473, Upstate Biotechnologies).

Cell lines, antibodies, drug treatments, FACS analysis, and luciferase reporter assay were performed as described in the Supplemental Material.

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