**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 Yu1,2,4

1Cancer Biology and Pharmacology, Genome Institute of Singapore, A*STAR (Agency for Science, Technology, and Research), Singapore 138672; 2Department 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-449a/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.

Supplemental material is available at http://www.genesdev.org.

Received May 8, 2009; revised version accepted September 1, 2009.

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 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-regulated miRNAs that directly target and affect E2F1 expression, miR-449a/b negatively regulate the E2F1 activity by targeting CDK6 and CDC25A towards 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 2388 GENES & DEVELOPMENT 23:2388–2393 © 2009 by Cold Spring Harbor Laboratory Press ISSN 0890-9369/09; www.genesdev.org
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 miRNA 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-449a/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 G1/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 (Roy 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].

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-449/a/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
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).

To confirm that these alterations on cell cycle regulation 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 queried the 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 mark 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 4. miR-449a/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 MCF10A cells treated with 100 nM control (NC) or miR-449a mimics. (C) MCF-7 cells were transfected with negative control (NC) or miR-449a mimics, followed by 100 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.
of examined DNA fragments are indicated by a, b, and c. (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 deacetylase (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 RNAmax 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.

Acknowledgments

We thank Dr. Kristian Helin for the ER-E2F1 plasmids. This work was supported by Agency for Science, Technology, and Research [A*Star] of Singapore.

References

Ambros V. 2004. The functions of animal microRNAs. Nature 431: 350–355.

Azuara V, Perry P, Sauer S, Spivakov M, Jorgensen HF, John RM, Gouti M, Casanova M, Warnes G, Merkenschlager M, et al. 2006. Chromatin signatures of pluripotent cell lines. Nat Cell Biol 8: 532–538.

Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, et al. 2006. A bivalient chromatin structure marks key developmental genes in embryonic stem cells. Cell 125: 1157–1168.

Chang TC, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH, Feldmann G, Yamakuchi M, Feltro L, Lowenstein CJ, et al. 2007. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Mol Cell 26: 745–752.

Esquela-Kerscher A, Slack FJ. 2006. Oncomirs—MicroRNAs with a role in cancer. Nat Rev Cancer 6: 259–269.

He L, He X, Lin LP, de Stanchina E, Xuan Z, Liang Y, Xue W, Zender L, Magnus J, Rudzon D, et al. 2007. A microRNA component of the p53 tumour suppressor network. Nature 447: 1130–1134.

Iliopoulos D, Bimpaki EI, Nesterova M, Stratakis CA. 2009. MicroRNA signature of primary pigmented nodular adreno cortical disease: Clinical correlations and regulation of Wnt signaling. Cancer Res 69: 3276–3282.

Jiang X, Tan J, Li J, Kivimae S, Yang X, Zhuang L, Lee PL, Chan MT, Yang X, Murthy Karuturi RK, Sun F, Aau M, Yu K, Shao R, Miller LD, Tan Pan ET, Yu Q. 2007. Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. Genes & Dev 21: 1050–1063.

Iliopoulos D, Bimpaki EL, Nesterova M, Stratakis CA. 2009. MicroRNA signature of primary pigmented nodular adreno cortical disease: Clinical correlations and regulation of Wnt signaling. Cancer Res 69: 3276–3282.

Jiang X, Tan J, Li J, Kivimae S, Yang X, Zhuang L, Lee PL, Chan MT, Stanton LW, Liu ET, et al. 2008. DACT3 is an epigenetic regulator of Wnt/β-catenin signaling in colorectal cancer and is a therapeutic target of histone modifications. Cancer Cell 13: 529–541.

Kang T, Wei Y, Honaker Y, Yamaguchi H, Appella E, Hung MC, Pimwana-Worms H. 2008. GSK-3β targets Cdc25A for ubiquitin-mediated proteolysis, and GSK-3β inactivation correlates with Cdc25A overproduction in human cancers. Cancer Cell 13: 36–47.

Lewis BP, Burge CB, Bartel DP. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120: 15–20.

Liang Y. 2008. An expression meta-analysis of predicted microRNA targets identifies a diagnostic signature for lung cancer. BMC Mol Genomics 1: 61. doi: 10.1186/1471-2164-9-61.

Lu C, Tej SS, Luo S, Haudenschild CD, Meyers BC, Green PJ. 2005. Elucidation of the small RNA component of the transcriptome. Science 309: 1567–1569.

Marson A, Levine SS, Cole MF, Frampton GM, Brambrink T, Johnstone S, Guenther MG, Johnston WK, Wernig M, Newman IE, et al. 2008. Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. Cell 134: 521–533.

Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, Giannoukos G, Alvarez P, Brockman W, Kim TK, Koche RP, et al. 2007. Genome-wide mapping of chromatin state in pluripotent and lineage-committed cells. Cell 125: 3553–3560.

Noonan EJ, Place RF, Pookot D, Basak S, Whitson JM, Hirata H, Giardina C, Dahiya R. 2009. miR-449a targets HDAC-1 and induces growth arrest in prostate cancer. Oncogene 28: 1714–1724.

O'Donnell KA, Wentzel EA, Zeller KL, Dang CV, Mendell JT. 2005. c-Myc-regulated microRNAs modulate E2F1 expression. Nature 435: 839–843.

Pan G, Tian S, Nie J, Yang C, Ruotti V, Wei H, Jonsdottir G, Stewart R, Thomson J. 2007. Whole-genome analysis of histone H3 lysine 4 and lysine 7 methylation in human embryonic stem cells. Cell Stem Cell 1: 299–312.

Petrocca F, Visone R, Onelli MR, Shah MH, Nicoloso MS, de Martino I, Iliopoulos D, Pilozzi E, Liu CG, Negrini M, et al. 2008. E2F1-regulated microRNAs impair TGFβ-dependent cell-cycle arrest and apoptosis in gastric cancer. Cancer Cell 13: 272–286.

Pickering MT, Stadler BM, Kowalik TF. 2009. miR-17 and miR-20a temper an E2F1-induced G1 checkpoint to regulate cell cycle progression. Oncogene 28: 140–145.

Raver-Shapira N, Marciano E, Meiri E, Spector Y, Rosenfeld N, Moskovits N, Bentwich Z, Oren M. 2007. Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. Mol Cell 26: 731–743.

Ray D, Terai Y, Furukawa PC, Ma ZQ, DeMayo FJ, Christov K, Heerema NA, Franks TS, Syi Y, Papoutsakis ET, et al. 2007. Deregulated CDC25A expression promotes mammary tumorigenesis with genomic instability. Cancer Res 67: 984–991.

Sherr CJ, McCormick F. 2002. The RB and p53 pathways in cancer. Cancer Cell 2: 103–112.

Sylvestre Y, De Guire V, Querido E, Mukhopadhyay UK, Bourvau D, Major F, Ferbeugy C, Chartrand P, et al. 2007. An E2F/miR-20a autoregulatory feedback loop. J Biol Chem 282: 2135–2143.

Tan J, Yang X, Zhuang L, Jiang X, Chen W, Lee PL, Karuturi RK, Tan PB, Liu ET, Yu Q. 2007. Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. Genes & Dev 21: 1050–1063.

Tempst P, Lin SP, Allis CD, et al. 2007. DNMT3L connects unmethylated CpG islands to the polycomb repressive complex 2 (PRC2) in mammalian cells. Nat Cell Biol 9: 532–538.

Wang Z, Pan H, Zhou J, Wang Y, Wang X, Basak S, Lutsky EJ, Gehrke EF, et al. 2007. A microRNA component of the p53 tumour suppressor network. Nature 447: 1130–1134.

Yiou E, Muller H, Prosperini E, Hateboer G, Cartwright P, Moroni MC, Helin K. 1999. CDC25A phosphatase is a target of E2F and is required for efficient E2F-induced S phase. Mol Cell Biol 19: 6379–6395.

Zeller KI, Dang CV, Mendell JT. 2005. c-Myc-regulated microRNAs modulate E2F1 expression. Nature 435: 839–843.
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, Min Feng, Xia Jiang, et al.

Genes Dev. 2009, 23:
Access the most recent version at doi:10.1101/gad.1819009